US005112749A

United States Patent [19]

Brey, III et al.

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[45] Date of Patent: May 12, 1992

[54] VACCINES FOR THE MALARIA CIRCUMSPOROZOITE PROTEIN

[75] Inventors: Robert N. Brey, III, Rochester; William R. Majarian, Pittsford; Subramonia Pillai, Rochester; Wayne T. Hockmeyer, Pittsford, all of N.Y.

[73] Assignee: Praxis Biologics, Inc., Rochester, N.Y.

[21] Appl. No.: 104,735

[22] Filed: Oct. 2, 1987

[51] Int. Cl.[5] C12N 15/00; C12N 1/21; C12N 1/00; C12P 21/02; A01N 63/00; C07H 15/12; C07K 3/00

[52] U.S. Cl. 435/172.3; 435/69.1; 435/257.3; 435/320.1; 435/879; 536/27; 530/350; 935/12; 935/27; 935/41; 935/56; 935/65; 935/72

[58] Field of Search 435/68, 70, 91, 172.1, 435/172.3, 320.1, 879, 69.1, 252.3; 424/88, 93; 530/350; 935/19, 27, 29, 41, 47, 56, 72; 536/27

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Primary Examiner—Joan Ellis

Attorney, Agent, or Firm—Hamilton, Brook, Smith & Reynolds

[57] ABSTRACT

The present invention is directed to attenuated strains of enteroinvasive bacteria that express a peptide or protein related to an epitope of the malaria parasites of the genus Plasmodium. The bacterial strains of the invention which can multiply in a host without causing significant disease or disorder, and which express a Plasmodium-related peptide that induces a protective immune response against malaria, can be used in live vaccine formulations for malaria. In specific embodiments, a Plasmodium-related peptide can be expressed as a fusion protein, for example, with a bacterial enterotoxin.

The invention also relates to methods for expression of malaria antigens or fragments thereof within attenuated enteroinvasive bacteria.

In particular embodiments, the invention is directed to the expression by attenuated Salmonella spp. of epitopes of Plasmodium circumsporozoite proteins.

50 Claims, 17 Drawing Sheets

ATT. DOC. NO. SL3261

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Taq I Nru I hinc II
AATTCGAAC CCCTTCGCGA AATACAGTCA ACAGATTACT TGCCGATGCT CCCGAAGGAA
Xmn I → CS gene sequence

AAAAAAATGA GAAAAAAAAC GAAAAAATAG AGCGTAATAA TAAATTGAAA CAACCACCAC

CACCACCAAA CCCAAATGAC CCACCACCAC CAAACCCAAA TGACCCACCA CCACCAAACC

CAAATGACCC ACCACCACCA AACGCAAATG ACCCAGCACC ACCAAACGCA AATGACCCAG

CACCACCAAA CGCAAATGAC CCAGCACCAC CAAACGCAAA TGACCCAGCA CCACCAAACG

CAAATGACCC AGCACCACCA AACGCAAATG ACCCAGCACC ACCAAACGCA AATGACCCAG

CACCACCAAA CGCAAATGAC CCACCACCAC CAAACCCAAA TGACCCAGCA CCACCACAAG

GAAATAACAA TCCACAACCA CAGCCACGGC CGCAGCCACA ACCACAGCCA CAGCCACAAC

CACAGCCACA GCCACAACCA CAGCCACGAC CACAGCCACA ACCACAGCCA GGTGGTAATA

ACAATAACAA AAATAATAAT AATGACGATT CTTATATCCC AAGCGCGGAA AAAATACTAG

AATTTGTTAA ACAGATCAGG GATAGTATCA CAGAGGAATG GTCTCAATGT AACGTAACAT

Xmn I
GTGGTTCTGG TATAAGAGTT AGAAAACGAA AAGGTTCAAA TAAGAAAGCA GAAGATTTGA

SspI
CCTTAGAAGA TATTGATACT GAAATTTGTA AAATGGATAA ATGTTCAAGT ATATTTAATA

SspI
TTGTAAGCAA TTCATTAGGA TTTGTAATAT TATTAGTATT AGTATTCTTT AATTAAATAA

SspI SspI
ACATTACGCA TGATTATAAA TATTTATATA TTATATAAAT ATTTTACATA CATATGACGT

GTGTAAACTT TATTTTTTTT ATTGTGAACT TTTCCTTATT TATTACGATT ATGTTTATAT

GTATATATAT TTAATATGTA AATCAAAAGA AAAAATAAAT AATAGAAGGC TTATTATATT

TATAATATAA ATTAAAAAAT AAAATATATA TGCATTACAA AATTTACTTT TTTTAGTTTA

Xmn I
TTTTTTTCGT GTTTATTATA TATGTAGTTA ACTTGCTATG ACGATATCGC GAAGGGGTTC
Nru I Toq I

GAATT

FIG. 1

```
CGA AAT ACA GTC AAC AGA TTA CTT GCC GAT GCT CCC GAA GGA AAA AAA AAT GAG AAA AAA
Arg Asn Thr Val Asn Arg Leu Leu Ala Asp Ala Pro Glu Gly Lys Lys Asn Glu Lys Lys

AAC GAA AAA ATA GAG CGT AAT AAT AAA TTG AAA CAA CCA CCA CCA CCA CCA AAC CCA AAT
Asn Glu Lys Ile Glu Arg Asn Asn Lys Leu Lys Gln Pro Pro Pro Pro Pro Asn Pro Asn
                               Region I
[GAC CCA CCA CCA CCA AAC CCA AAT][GAC CCA CCA CCA CCA AAC CCA AAT][GAC CCA CCA CCA
[Asp Pro Pro Pro Pro Asn Pro Asn][Asp Pro Pro Pro Pro Asn Pro Asn][Asp Pro Pro Pro

CCA AAC GCA AAT][GAC CCA GCA CCA CCA AAC GCA AAT][GAC CCA GCA CCA CCA AAC GCA AAT]
Pro Asn Ala Asn][Asp PRo Ala Pro Pro Asn Ala Asn][Asp Pro Ala Pro Pro Asn Ala Asn]

[GAC CCA GCA CCA CCA AAC GCA AAT][GAC CCA GCA CCA CCA AAC GCA AAT][GAC CCA GCA CCA
[Asp Pro Ala Pro Pro Asn Ala Asn][Asp Pro Ala Pro Pro Asn Ala Asn][Asp Pro Ala Pro

GCA AAC GCA AAT][GAC CCA GCA CCA CCA AAC GCA AAT][GAC CCA GCA CCA CCA AAC GCA AAT]
Pro Asn Ala Asn][Asp Pro Ala Pro Pro Asn Ala Asn][Asp Pro Ala Pro Pro Asn Ala Asn]

GAC CCA CCA CCA CCA AAC CCA AAT GAC CCA GCA CCA CCA CAA GGA AAT AAC AAT [CCA CAA]
Asp Pro Pro Pro Pro Asn Pro Asn Asp Pro Ala Pro Pro Gln Gly Asn Asn Asn [Pro Gln]

[CCA CAG][CCA CGG][CCG CAG][CCA CAA][CCA CAG][CCA CAG][CCA GAA][CCA CAG][CCA CAG][CCA CAA]
[Pro Gln][Pro Arg][Pro Gln][Pro Gln][Pro Gln][Pro Gln][Pro Gln][Pro Gln][Pro Gln][Pro Gln]

CCA CAG CCA CGA CCA CAG CCA CAA CCA CAG CCA GGT GGT AAT AAC AAT AAC AAA AAT AAT
Pro Gln Pro Arg Pro Gln Pro Gln Pro Gln Pro Gly Gly Asn Asn Asn Asn Lys Asn Asn

AAT AAT GAC GAT TCT TAT ATC CCA AGC GCG GAA AAA ATA GTA GAA TTT GTT AAA CAG ATC
Asn Asn Asp Asp Ser Tyr Ile Pro Ser Ala Glu Lys Ile Leu Glu Phe Val Lys Gln Ile

AGG GAT AGT ATC ACA GAG GAA TGG TCT CAA TGT AAC GTA ACA TGT GGT TCT GGT ATA AGA
Arg Asp Ser Ile Thr Glu Glu Trp Ser Gln Cys Asn Val Thr Cys Gly Ser Gly Ile Arg
                                Region II
GTT AGA AAA CGA AAA GGT TCA AAT AAG AAA GCA GAA GAT TTG ACC TTA GAA GAT ATT GAT
Val Arg Lys Arg Lys Gly Ser Asn Lys Lys Ala Glu Asp Leu Thr Leu Glu Asp Ile Asp

ACT GAA ATT TGT AAA ATG GAT AAA TGT TCA AGT ATA TTT AAT ATT GTA AGC AAT TCA TTA
Thr Glu Ile Cys Lys Met Asp Lys Cys Ser Ser Ile Phe Asn Ile Val Ser Asn Ser Leu

GGA TTT GTA ATA TTA TTA GTA TTA GTA TTC TTT AAT TAA ATA AAC ATT ACG CAT GAT TAT
Gly Phe Val Ile Leu Leu Val Leu Val Phe Phe Asn

AGA TAT TTA TAT ATT ATA TAA ATA TTT TAC ATA CAT ATG ACG TGT GTA AAC TTT ATT TTT
```

FIG.2

```
                                                    ┌ SIGNAL SEQUENCE
EcoRI
AAT TCG GGA TGA ATT ATG AAT AAA GTA AAA TTT TAT GTT TTA TTT ACG GCG TTA CTA TCC
      S/D           Met Asn Lys Val Lys Phe Tyr Val Leu Phe Thr Ala Leu Leu Ser
                  SacI ┌ Mature LT-B
TCT CTA TGT GCA CAC GGA GCT CCT CAG TCT ATT ALA GAA CTA TGT TCG GAA TAT CAC AAC
Ser Leu Cys Ala His Gly Ala Pro Gln Ser Ile Thr Glu Leu Cys Ser Glu Tyr His Asn
                                                          Cla I
ACA CAA ATA TAT ACG ATA AAT GAC AAG ATA CTA TCA TAT ACG GAA TCG ATG GCA GGC AAA
Thr Gln Ile Tyr Thr Ile Asn Asp Lys Ile Leu Ser Tyr Thr Glu Ser Met Ala Gly Lys
                                                                Xma I
AGA GAA ATG GTT ATC ATT ACA TTT AAG AGC GGC GCA ACA TTT CAG GTC GAA GTC CCG GGC
Arg Glu Met Val Ile Ile Thr Phe Lys Ser Gly Ala Thr Phe Gln Val Glu Val Pro Gly

AGT CAA CAT ATA GAC TCC CAA AAA AAA GCC ATT GAA AGG ATG AAG GAC ACA TTA AGA ATC
Ser Gln His Ile Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg Ile

ACA TAT CTG ACC GAG ACC AAA ATT GAT AAA TTA TGT GTA TGG AAT AAT AAA ACC CCC AAT
Thr Tyr Leu Thr Glu Thr Lys Ile Asp Lys Leu Cys Val Trp Asn Asn Lys Thr Pro Asn
                                  Spe I
TCA ATT GCG GCA ATC AGT ATG GAA AAC TAG TTT GCT TTA AAA GCA TGT CTA ATG CTA GGA
Ser Ile Ala Ala Ile Ser Met Glu Asn

ACC TAT ATA ACA ACT ACT GTA CTT ATA CTA ATG AGC CTT ATG CTG CAT TTG AAA AGG CGG

TAG AGG ATG CAA TAC CGA TCC TTA AAC TGT AAC ACT ATA ACA GCT TCC ACT ACA GGG AGC
                                                          Hind III
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```
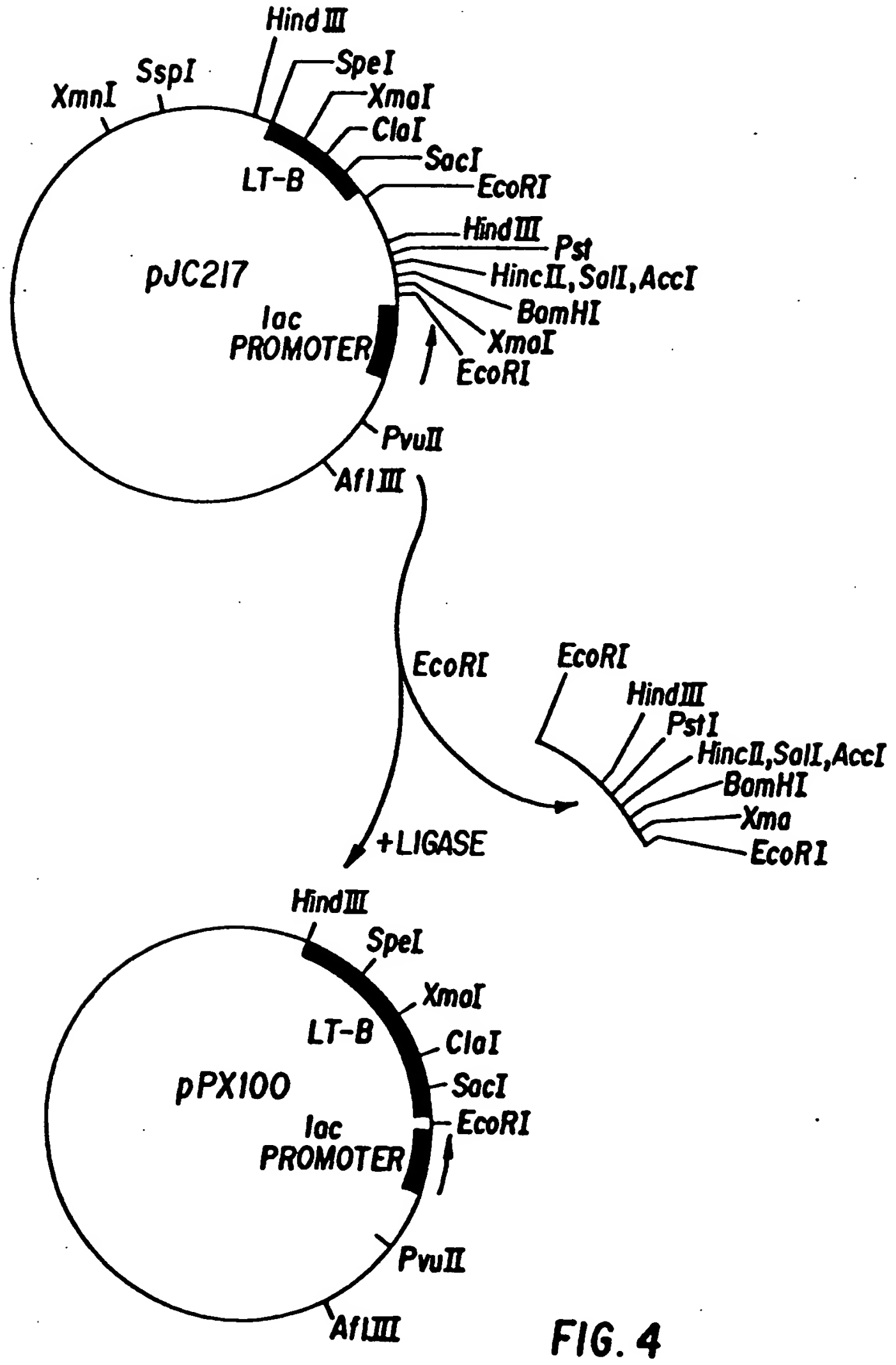

FIG. 3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SpeI
XmaI
ClaI
SacI
EcoRI
HindIII
Pst
HincII,SalI,AccI
BamHI
XmaI
EcoRI
PvuII
AflIII
XmnI
SspI
LT-B
pJC217
lac
PROMOTER
EcoRI
EcoRI
HindIII
PstI
HincII,SalI,AccI
BamHI
Xma
EcoRI
+LIGASE
HindIII
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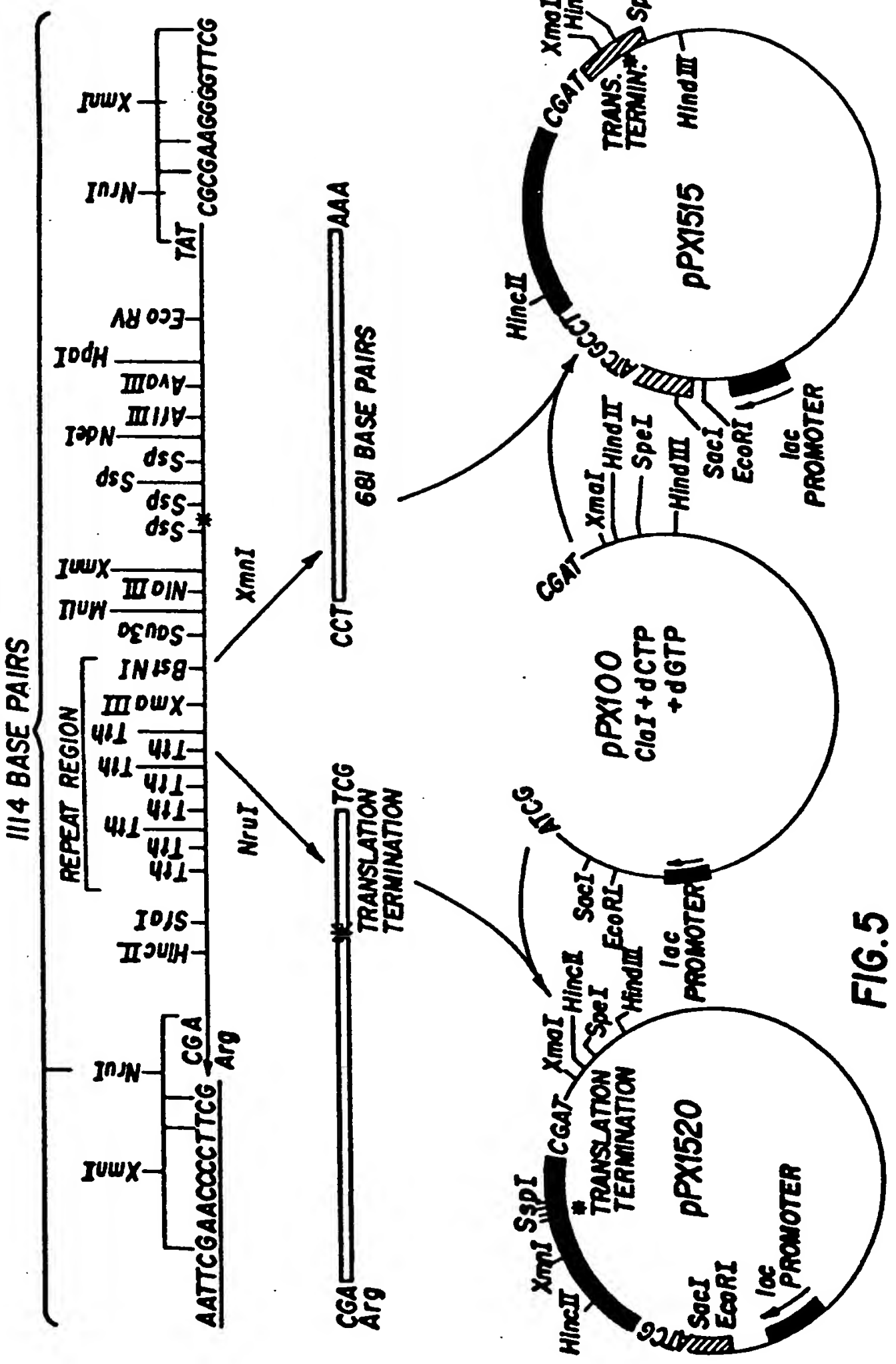
AflIII
LT-B
pPX100
lac
PROMOTER

FIG. 4


1114 BASE PAIRS
REPEAT REGION
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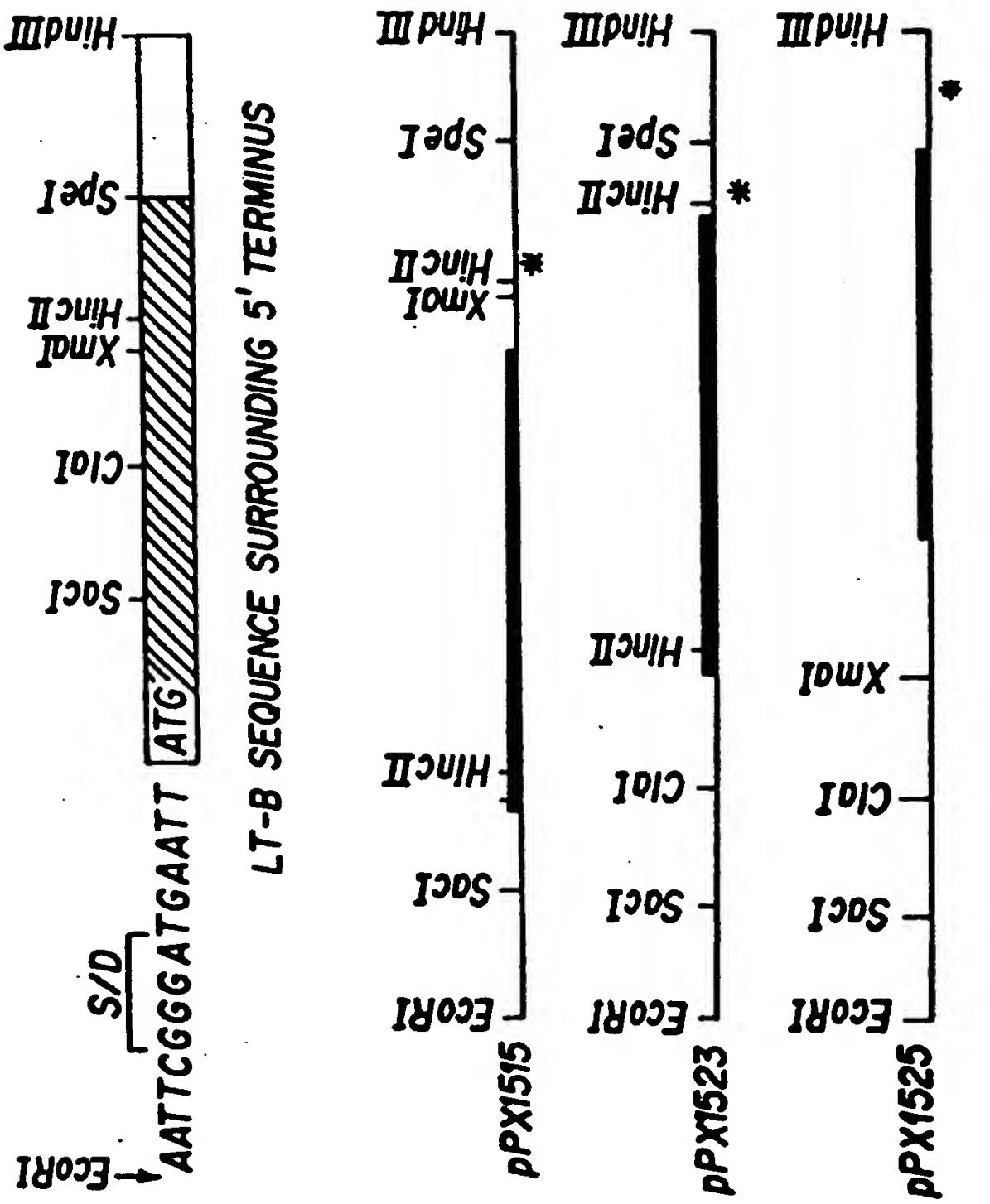
NruI
HincII
SfaI
TIR
TIR
TIR
TIR
TIR
TIR
TIR
XmaIII
MaeII
SauIIIa
BstNI
XmnI
NIaIII
Sap
Sap
Sap
NdeI
AlwIII
AvaIII
HpaI
EcoRV
TAT
CGCGAAGGGGTTCG
NruI
XmaI
AATTCGAACCCCTTCG
CGA
Arg
CCT
681 BASE PAIRS
AAA
CGA
Arg
TCG
TRANSLATION
TERMINATION
pPX1515
ATCGCCT
HincII
CGAT
TRANS.
TERMIN.
XmaI
HincII
SpeI
HindIII
SacI
EcoRI
lac
PROMOTER
pPX100
ClaI + dCTP
+ dGTP
pPX1520
ATCG
XmnI
SspI
HincII
CGAT
TRANSLATION
TERMINATION
SacI
EcoRI
lac
PROMOTER

FIG.5


HindIII
SpeI
HincII
XmaI
ClaI
SacI
S/D
ATG
AATTCGGGATGAATT
EcoRI
LT-B SEQUENCE SURROUNDING 5' TERMINUS
HindIII
SpeI
XmaI
HincII*
HincII
SacI
EcoRI
pPX1515
HindIII
SpeI
HincII*
HincII
ClaI
SacI
EcoRI
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SacI
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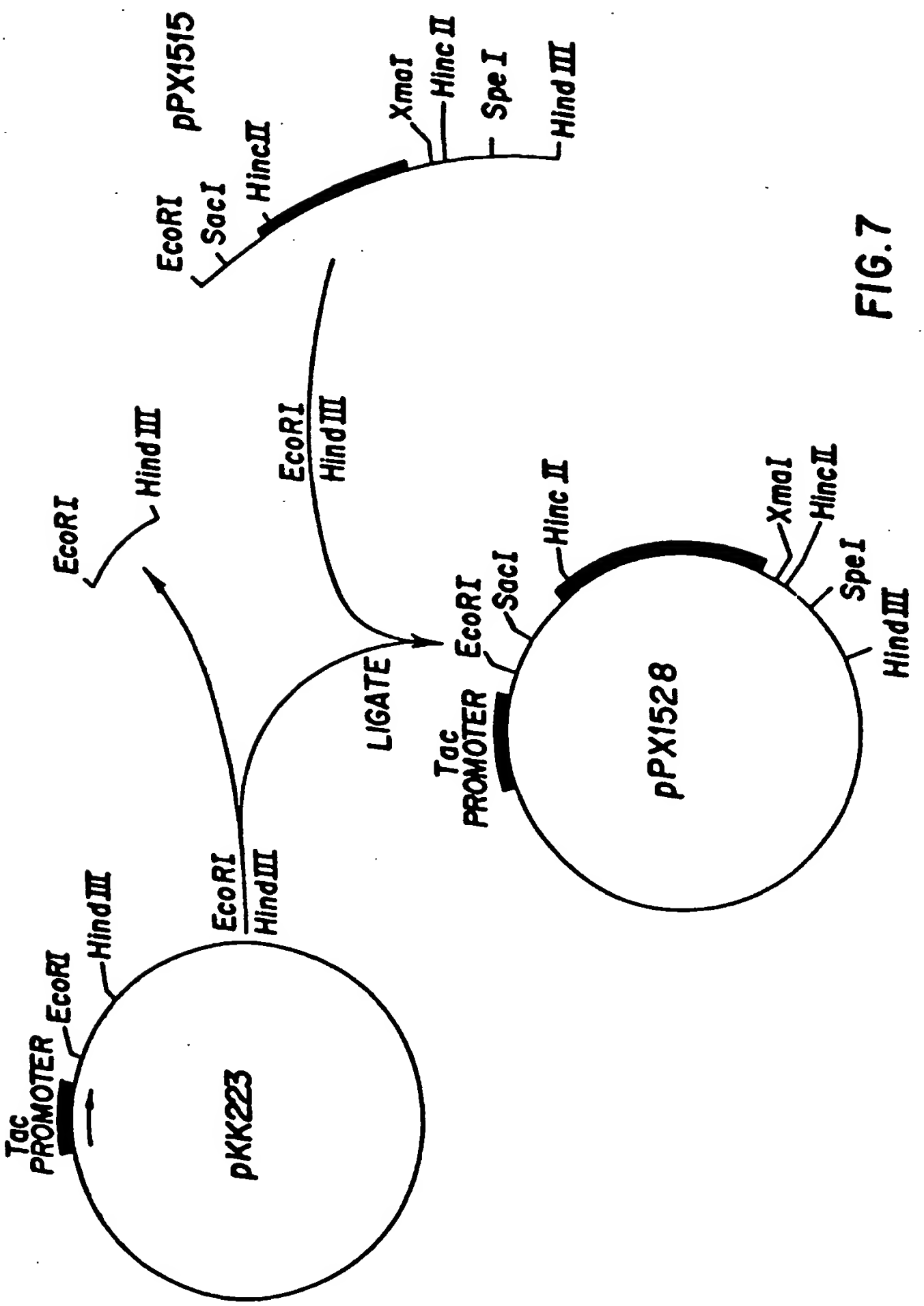
FIG. 6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SpeI
HindIII
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HindIII
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LIGATE
EcoRI
SacI
HincII
XmaI
HincII
SpeI
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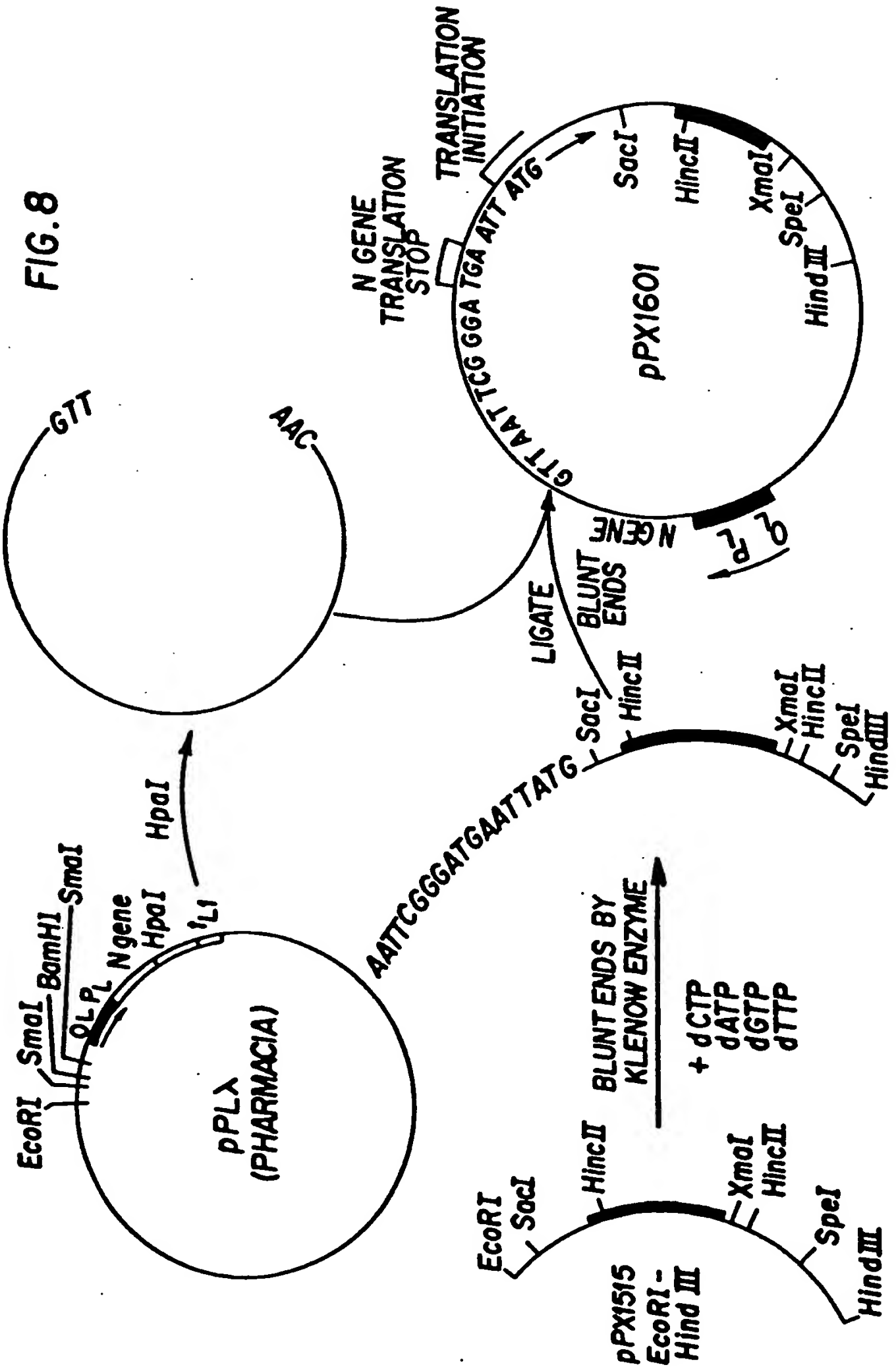
Tac
PROMOTER
EcoRI
HindIII
pKK223

FIG. 7

FIG. 8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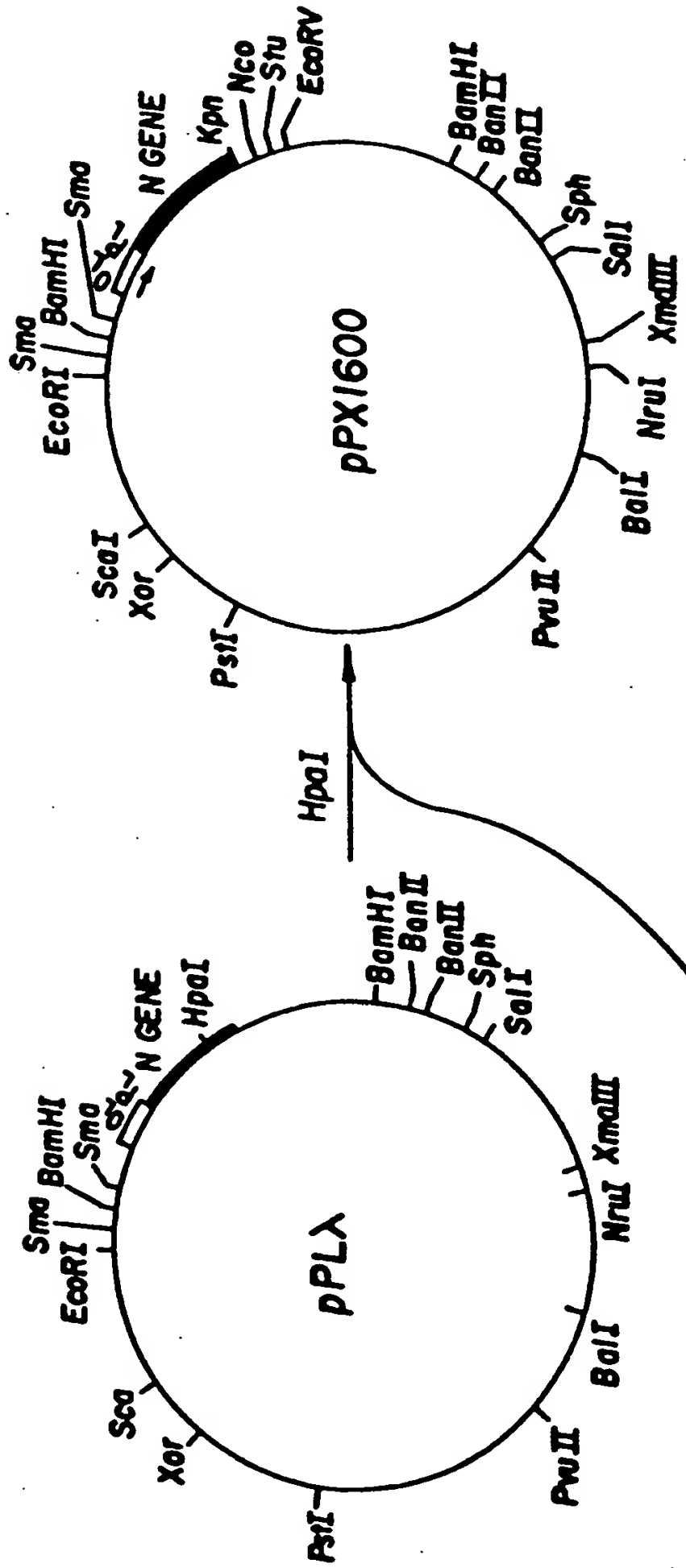
SacI
HincII
XmaI
SpeI
HindIII
N GENE
QL PL
GTT
AAC
LIGATE
BLUNT ENDS
AATTCGGATGAATTATG
SacI
HincII
XmaI
HincII
SpeI
HindIII
pPLλ (PHARMACIA)
EcoRI
SmaI
BamHI
QL PL
N gene
HpaI
tL1
HpaI
BLUNT ENDS BY KLENOW ENZYME
+ dCTP
dATP
dGTP
dTTP
EcoRI
SacI
HincII
XmaI
HincII
SpeI
HindIII
pPX1515 EcoRI-Hind III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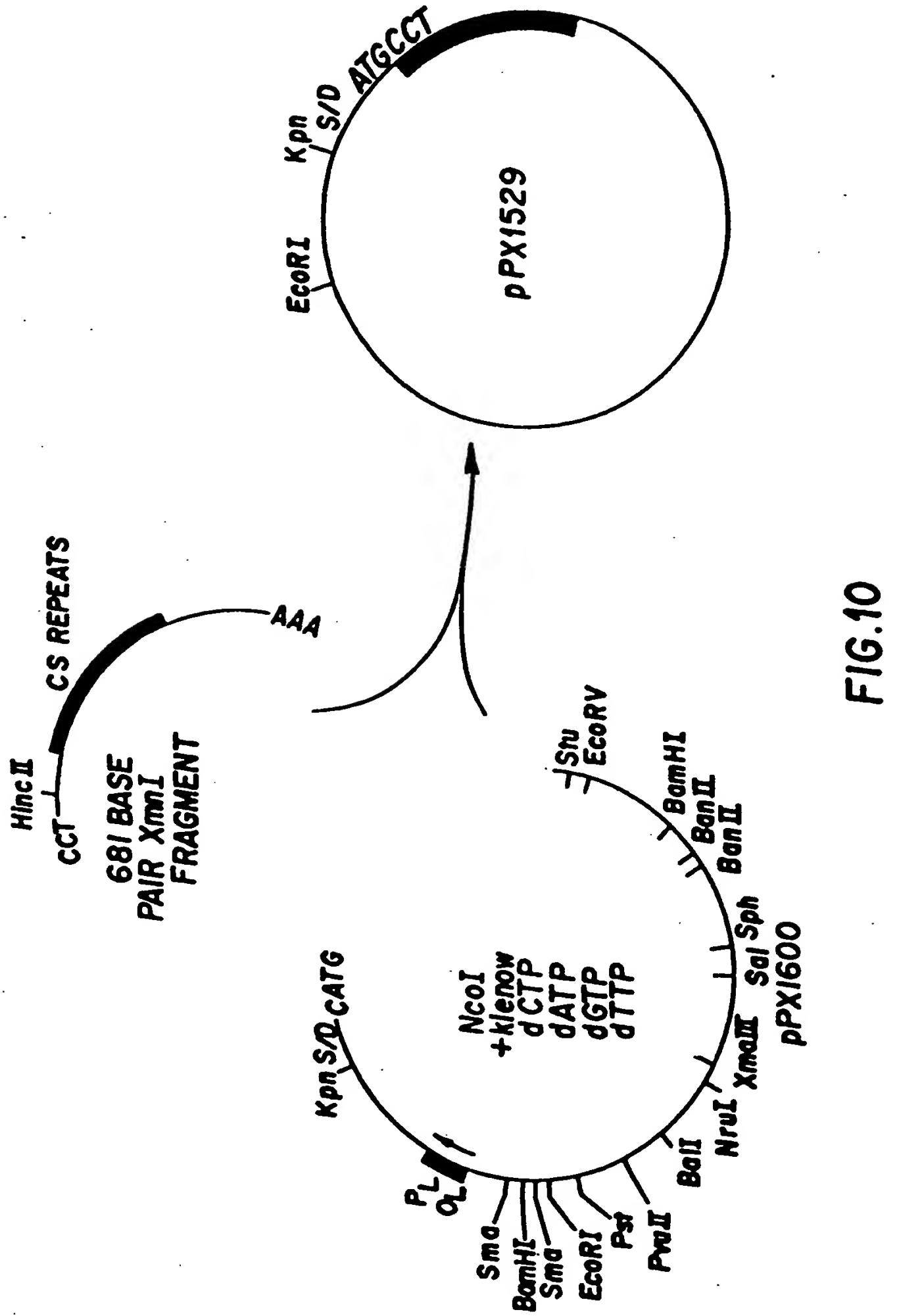
Sma
EcoRI
BamHI
Sma
Sd-
N GENE
Kpn
Nco
Stu
EcoRV
BamHI
BanII
BanII
Sph
SalI
XmaIII
NruI
BalI
PvuII
PstI
Sca
Xor
HpaI
pPLλ
Sma
EcoRI
BamHI
Sma
Sd-
N GENE
HpaI
BamHI
BanII
BanII
Sph
SalI
NruI
XmaIII
BalI
PvuII
PstI
Sca
Xor
SYNTHETIC OLIGONUCLEOTIDE
GGT ACC TAA CTA AGG AGG TTT ACC CAT GGA GGC CTT
KpnI * NcoI StuI
SYNTHETIC SHINE/DALGARNO SEQUENCE
GAT ATC TGA ATG ACT GA
EcoRV * * * *

FIG. 9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EcoRI
AAA
CS REPEATS
HincII
CCT
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PAIR XmnI
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NcoI
+klenow
dCTP
dATP
dGTP
dTTP
Kpn S/D CATG
PL
OL
Sma
BamHI
Sma
EcoRI
Pst
PvuII
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XmaIII
Sal Sph
pPX1600
Stu
EcoRV
BamHI
BanII
BanII

FIG.10

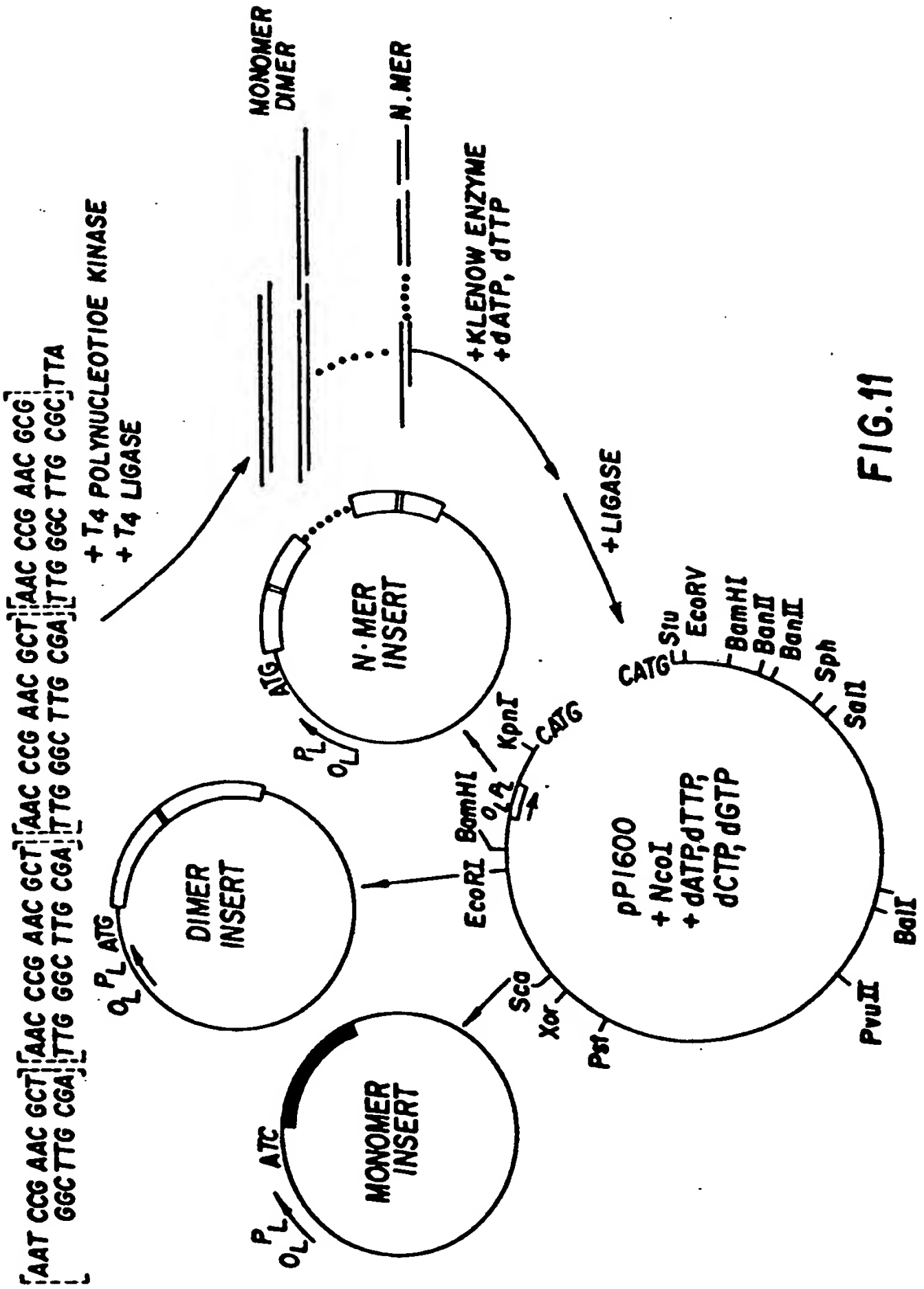
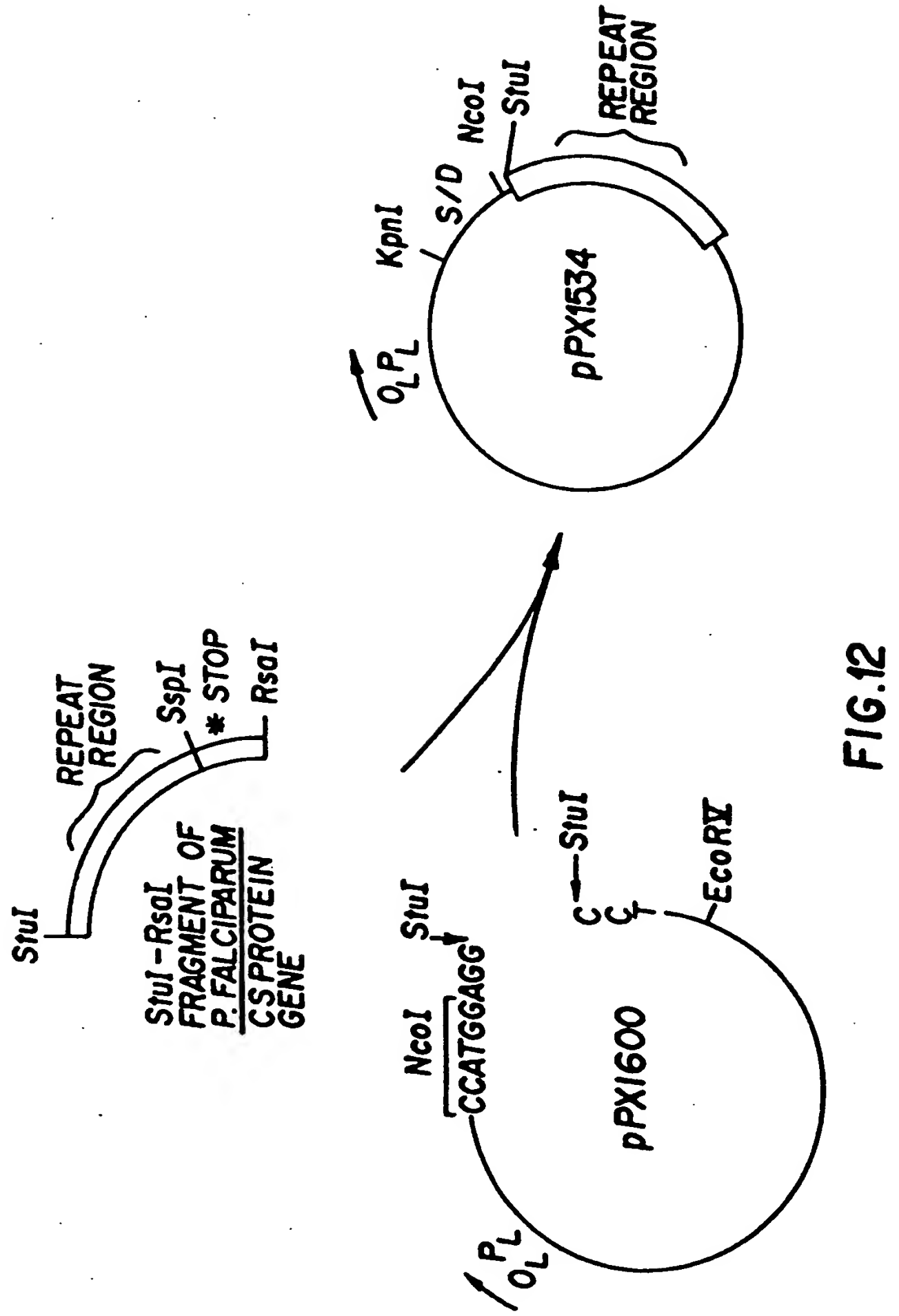

+ T4 POLYNUCLEOTIOE KINASE
+ T4 LIGASE
MONOMER DIMER
N.MER
+KLENOW ENZYME +dATP, dTTP
+LIGASE
N·MER INSERT
DIMER INSERT
MONOMER INSERT
pP1600 + NcoI + dATP,dTTP, dCTP, dGTP
EcoRI BamHI KpnI CATG
Sca Xor Pst PvuII BalI
CATG Sfu EcoRV BamHI BanII BanII Sph SalI

FIG.11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S/D
NcoI
StuI
REPEAT REGION
StuI
REPEAT REGION
SspI
* STOP
RsaI
StuI-RsaI FRAGMENT OF P. FALCIPARUM CS PROTEIN GENE
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C
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StuI
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$O_L P_L$

FIG.12

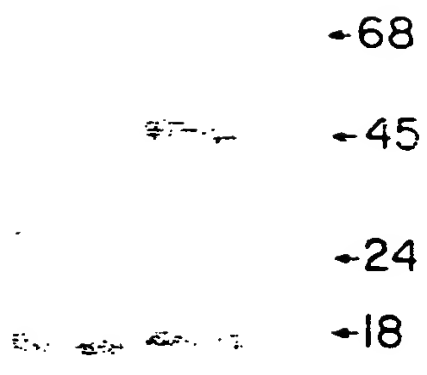

FIG. 13

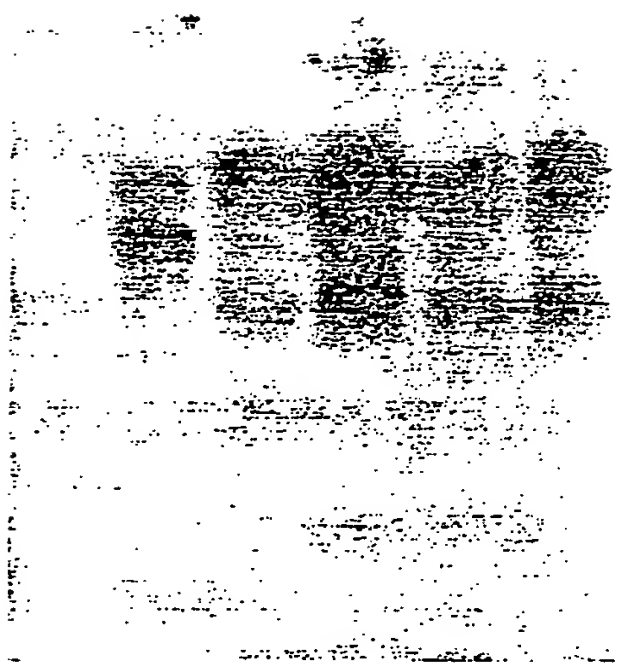

FIG. 14

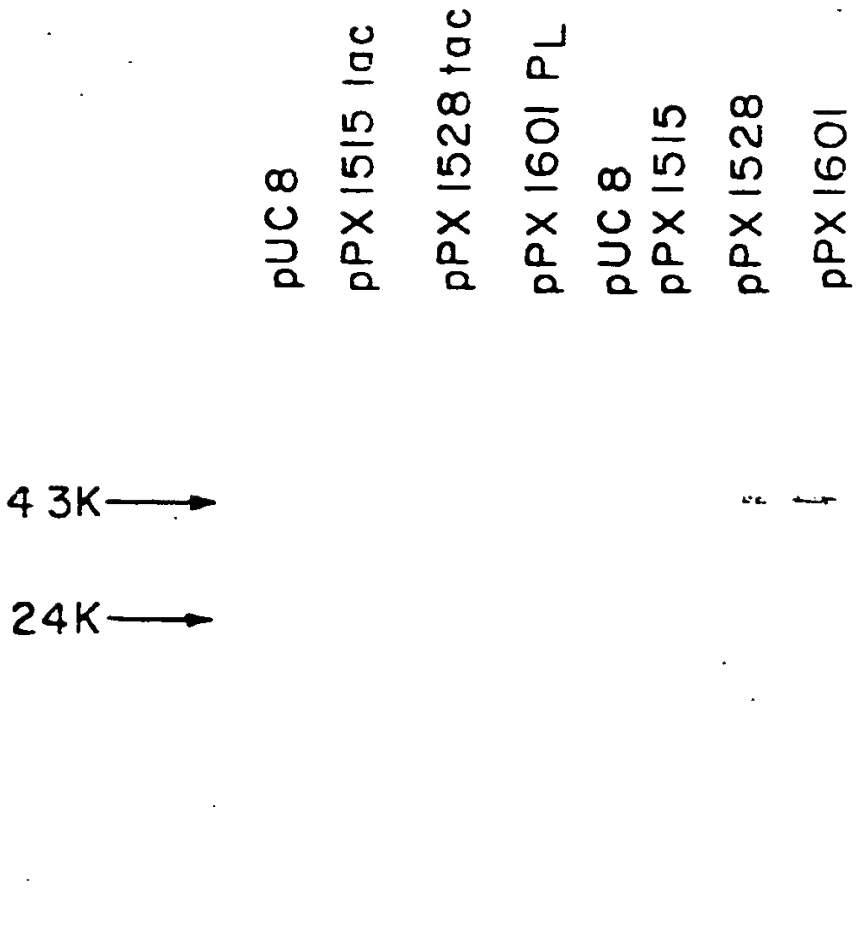

FIG. 15A

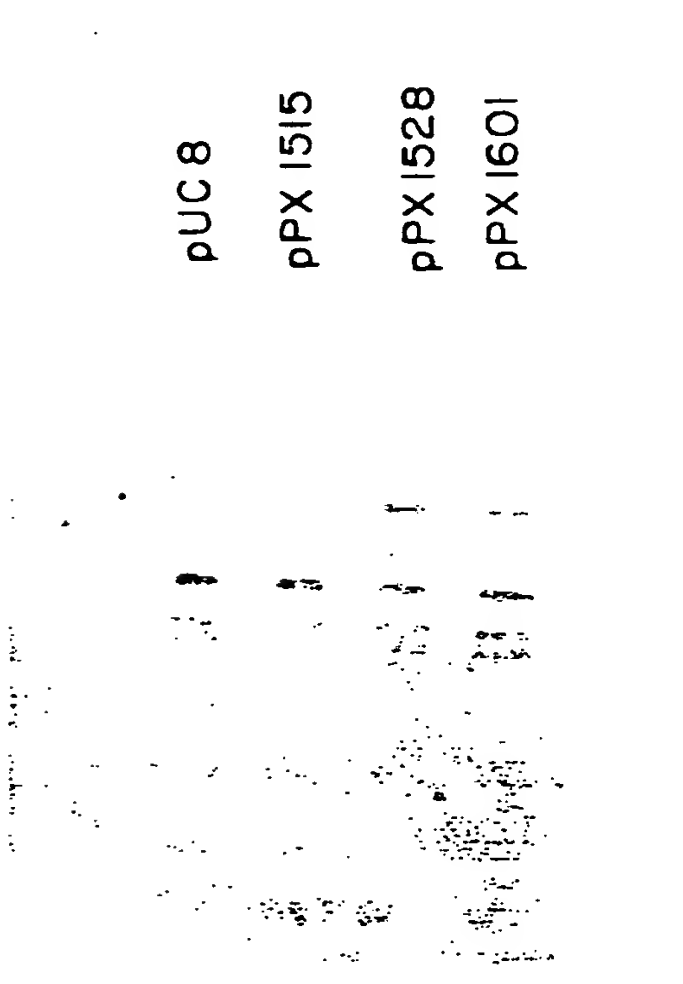

FIG. 15B

S. typhi TY523

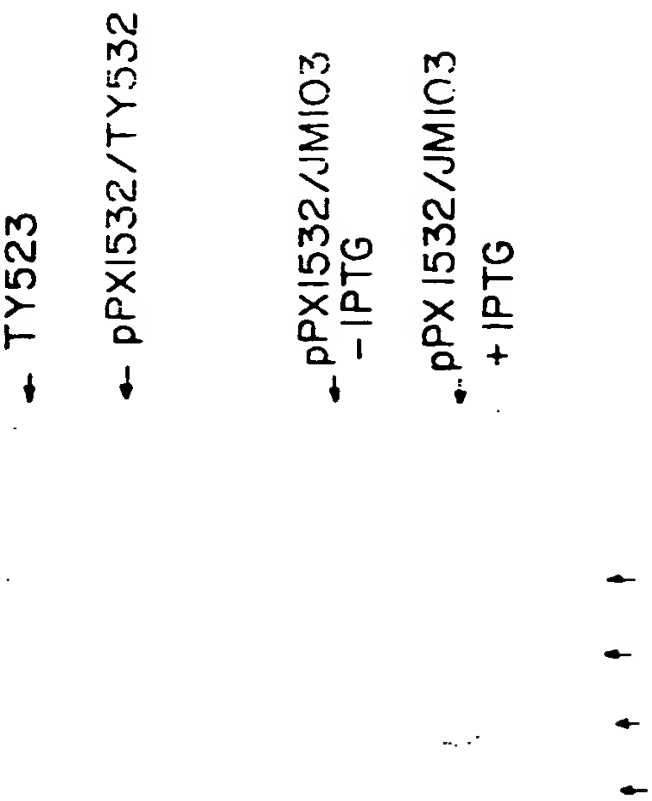
TY523
pPX1532/TY532
pPX1532/JM103 -IPTG
pPX1532/JM103 +IPTG
SL1438
2/SL1438
SL3261
2/L3261

FIG. 16A

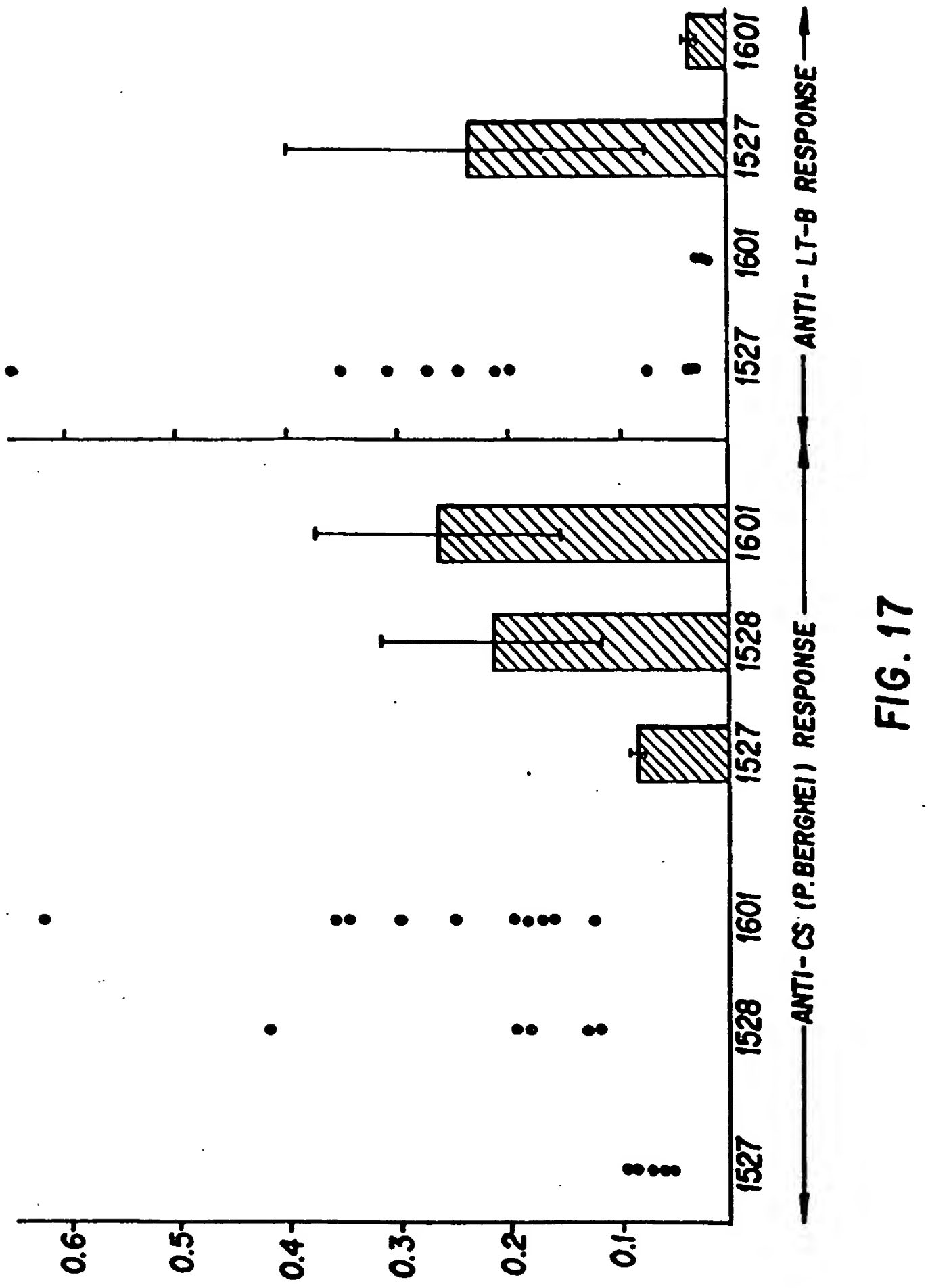
1601
1527
1601
1527
1601
1528
1527
1601
1528
1527
ANTI-LT-B RESPONSE
ANTI-CS (P.BERGHEI) RESPONSE
0.6
0.5
0.4
0.3
0.2
0.1

FIG. 17

VACCINES FOR THE MALARIA CIRCUMSPOROZOITE PROTEIN

1. FIELD OF THE INVENTION

The present invention is directed to attenuated strains of enteroinvasive bacteria that express peptides and proteins related to epitopes of the malaria parasites of the genus Plasmodium. The bacterial strains of the present invention which can multiply in a host without causing significant disease or disorder, and which express Plasmodium-related peptides that induce a protective immune response against malaria, can be used in live vaccine formulations for malaria. Such vaccine formulations can be univalent or multivalent.

In particular, the vaccine vector strains of the present invention comprise attenuated Salmonella bacteria which retain their enteroinvasive properties but lose in large part their virulence properties.

In a preferred embodiment of the invention, a gene or gene fragment encoding all or part of the circumsporozoite malaria antigen can be expressed in Salmonella bacteria that have been attenuated by chromosomal deletion of gene(s) for aromatic compound biosynthesis, for use as a live vaccine for malaria.

2. BACKGROUND OF THE INVENTION

2.1. RECOMBINANT DNA TECHNOLOGY AND GENE EXPRESSION

Recombinant DNA technology involves insertion of specific DNA sequences into a DNA vehicle (vector) to form a recombinant DNA molecule which is capable of replication in a host cell. Generally, the inserted DNA sequence is foreign to the recipient DNA vehicle, i.e., the inserted DNA sequence and the DNA vector are derived from organisms which do not exchange genetic information in nature, or the inserted DNA sequence may be wholly or partially synthetically made. Several general methods have been developed which enable construction of recombinant DNA molecules.

Regardless of the method used for construction, the recombinant DNA molecule must be compatible with the host cell, i.e., capable of autonomous replication in the host cell or stably integrated into one or more of the host cell's chromosomes. The recombinant DNA molecule should preferably also have a marker function which allows the selection of the desired recombinant DNA molecule(s). In addition, if all of the proper replication, transcription, and translation signals are correctly arranged on the recombinant vector, the foreign gene will be properly expressed in, e.g., the transformed bacterial cells, in the case of bacterial expression plasmids, or in permissive cell lines or hosts infected with a recombinant virus or carrying a recombinant plasmid having the appropriate origin of replication.

Different genetic signals and processing events control levels of gene expression such as DNA transcription and messenger RNA (mRNA) translation. Transcription of DNA is dependent upon the presence of a promoter, which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and furthermore, procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals, which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno (S/D) sequence (Shine, J. and Dalgarno, L., 1975, Nature 254:34–38) on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The S/D sequences are complementary to the 3' end of the 16S rRNA (ribosomal RNA), and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome (id.).

Successful expression of a cloned gene requires sufficient transcription of DNA, translation of the mRNA and in some instances, post-translational modification of the protein. Expression vectors have been used to express genes under the control of an active promoter in a suitable host, and to increase protein production.

2.2. VACCINATION AGAINST MALARIA

A global public health goal is the control and eventual eradication of human malaria. It is estimated that over 500 million people in tropical regions are exposed to malaria annually, and 1.5 to 2 million people die from this disease (Sturchler, D., 1984, Experientia 40:1357). Efforts to control malaria have historically focussed on control of the mosquito vector and the development of antimalarial drugs. These efforts have met with only limited success. New prophylactic and therapeutic drugs are of limited effectiveness because drug-resistant strains can appear rapidly in endemic areas. Control of the mosquito vector depends largely upon implementation of insecticide-based control programs which, due to cost and other factors, are difficult to maintain in developing nations. Vector resistance to modern insecticides has compounded the problem, and resulted once again in the resurgence of malaria.

Mammalian hosts can be infected by the sporozoite form of the malaria parasite, which is injected by the female Anopheles mosquito during feeding. Sporozoites injected into the bloodstream are carried rapidly to the liver where they invade hepatocytes. Once in hepatocytes, sporozoites develop into merozoite forms, which are released from hepatocytes and invade erythrocytes. Within the erythrocyte, the parasite asexually reproduces, from rings to schizonts. The mature schizont contains merozoites which, upon rupture of the erythrocyte, can invade other erythrocytes, causing clinical manifestations of the disease. Some merozoites differentiate into sexual forms, called gametocytes, which are taken up by mosquitoes during a blood meal. After fertilization of gametocytes in the mosquito midgut, developing ookinetes can penetrate the gut wall and encyst. Rupture of such oocysts allows release of sporozoites which migrate to the salivary glands to be injected when the female mosquito takes another blood meal, thus completing the infectious cycle.

Experiments conducted in the 1960s demonstrated that vaccination with X-irradiated sporozoites of *P. berghei* protected mice against sporozoite challenge which was lethal in unvaccinated animals (Nussenzweig, R., et al., 1969, Mil. Med. 134:1176). This observation was later extended to clinical studies in humans, where immunization with X-irradiated sporozoites of *P.*

falciparum or *P. vivax* protected human volunteers against sporozoite challenge delivered through the bites of infected mosquitoes (Clyde. D. F., et al., 1975. Am. J. Trop. Med. Hyg. 24:397; Rieckmann. K. H., et al., 1979. Bull. WHO 57:261). This protection was thought to be mediated by antibody. Serum from immunized animals. including humans. formed a precipitate around the surface of live. mature sporozoites. This reaction has been termed the circumsporozoite precipitin (CSP) reaction. These same sera blocked the ability of sporozoites to invade human hepatoma cells in culture (ISI assay) (Hollingdale. M. R., et al., 1984. J. Immunol. 132:909). In other studies. a single antigenic determinant localized on the surface of *P. berghei* sporozoites. termed the circumsporozoite protein. was identified. It was shown that a monoclonal antibody reacting with the circumsporozoite (CS) protein of *P. berghei* could passively transfer immunity to recipient animals. These animals were protected from sporozoite challenge in a dose-dependent fashion (Potocnjak. P. N., et al., 1980. J. Exp. Med. 151:1504). Evidence also existed that cell-mediated immunity was important (Chen. D. H., et al., 1977. J. Immunol. 118:1322; Verhave. J. P., et al., 1978. J. Immunol 121:1031).

The first CS protein gene to be cloned was derived from the H strain of *P. knowlesi*. a simian parasite (Ozaki et al., 1983. Cell 34:815). The genes encoding the CS proteins of the human malaria parasites *P. falciparum* (Dame et al., 1984. Science 225:593). *P. vivax* (McCutchan et al., 1984. Science 230:1381). the simian parasite *P. cynomolgi* (Enea et al., 1984. Science 225:628). and the rodent parasite *P. berghei* (Weber et al., 1987. Exp. Parasitol. 63:295) were also cloned and sequenced. A characteristic feature of the CS genes of each of the parasites is a central region which encodes over one-third of the protein. containing a series of repeated peptide sequences. The primary amino acid sequence. the length of the repeated sequence. and the number of repeats vary with each species of parasite. The repeat region epitopes are characteristic of each species. The gene encoding the CS protein of *P. falciparum* specifies a central repeat region of a tetrapeptide (asn-ala-asn-pro) repeated 37 times. interrupted in four locations by the nonidentical tetrapeptide (asn-val-asp-pro). The central repeat region of *P. vivax* CS protein contains 19 nonapeptides; the central sequence of *P. knowlesi* contains 12 dodecapeptides. and the repeat region of *P. berghei* contains 12 octapeptides. Comparison of sequences from *P. knowlesi* (H strain) and *P. falciparum* and *P. vivax* reveals no sequence homology except for two short amino acid sequences flanking the repeat region, termed Region I and Region II.

Efforts to develop an effective anti-sporozoite vaccine for *P. falciparum* have used peptides derived from the circumsporozoite (CS) repeat region and the two flanking Region I and Region II sequences (Ballou, W. R., et al., 1985, Science 228:996). These experiments showed that antibody to the repeat region but not to the conserved sequences recognized authentic CS protein, produced CSP activity, and blocked sporozoite invasion (ISI) in vitro. A recombinant DNA subunit vaccine composed of 32 *P. falciparum* tetrapeptide repeats fused to 32 amino acids of the tetracycline resistance gene was produced in *E. coli* (Young, J. F., et al., 1985, Science 228:958) Likewise, a peptide-carrier vaccine composed of three repeats of the peptide asn-ala-asn-pro (NANP) conjugated to tetanus toxoid was developed (Zavala. F., et al., 1985. Science 228:1436). In each case. preclinical studies indicated that biologically active (as shown by CSP and ISI) anti-sporozoite antibodies were elicited as a result of immunization (Ballou. R., et al., 1987. The Lancet 1:1277; Herrington et al., 1987. Nature 328:257). Human safety and immunogenicity studies with both vaccines yielded similar results. Both vaccine preparations were well tolerated at doses ranging from 10 micrograms to 800 micrograms. and both elicited some anti-CS antibodies in all immunized subjects. However. high titers were not achieved. In addition. subsequent booster immunizations with the peptide-carrier vaccine did not result in increased antibody titers. Several individuals from each study were then challenged with live sporozoites in order to test the efficacy of these vaccine preparations. Once again, similar results were achieved with both vaccines; the level of protection (as measured by a delay in the appearance of blood stage parasites) correlated with the anti-CS antibody titers of the challenged individuals, but in each trial, only one individual was protected. Parallel studies to evaluate the feasibility of human subunit vaccine development have been examined in the rodent *P. berghei* malaria model (Egan et al., 1987. Science 236:453).

A recent study has reported that levels of naturally acquired antibodies to the *P. falciparum* CS protein, as high as those achieved by a subunit sporozoite vaccine (Ballou, W. R., et al., 1987, Lancet 1987-1:1277). did not protect against *P. falciparum* infection during a 98-day interval in a malaria-endemic area.

In different studies. subunit vaccines containing peptides of other *P. falciparum* antigens have been investigated (Patarroyo. M. E., et al., 1987. Nature 629–632; Cheung. A., et al., 1986. Proc. Natl. Acad. Sci. U.S.A. 83:8328–8332; Collins. W. E., et al., 1986. Nature 323 259–262). In addition. recombinant vaccinia viruses which express *P. falciparum* antigens have been described for use (PCT International Publication Number WO 87/01386, published Mar. 12. 1987).

Perspectives and recent advances in malaria vaccination have been described (Miller. L. H., et al., 1984, Phil. Trans. R. Soc. Lond. B307:99–115; 1985, Vaccines87. Channock et al., eds., Cold Spring Harbor Laboratory. Cold Spring Harbor. N.Y. pp. 81–106, 117–124).

2.3. BACTERIA OF THE GENUS SALMONELLA

Bacteria of the genus Salmonella include over 2,000 serotypes, many of which are capable of causing enteric disease in man and animals. Of the diseases most frequently associated with Salmonella outbreak, typhoid fever is notable for its severity and high mortality. In humans, typhoid (or enteric) fever results from invasion and dissemination of *S. typhi*, although other members of the Salmonella are capable of invading and localizing in organ tissues, causing less severe symptoms. In other outbreaks of salmonellosis (such as food poisoning), the disease is non-invasive and confined to symptoms of gastroenteritis, and in humans, the disease is not associated with *S. typhi*. In animals, outbreaks of typhoid fever may be associated with numerous serotypes, e.g., *S. choleraesuis* in swine, *S. gallinarum* in poultry, and *S. dublin* and *S. typhimurium* in cattle (Topley and Wilson's Principles of Bacteriology, Virology, and Immunity, 6th ed., Williams & Wilkins Co., Baltimore, Md.). The host specificity and severity of the resultant disease varies among the serotypes of Salmonella.

The first instance of an attenuated oral vaccine for typhoid fever in humans was a streptomycin-dependent

S. typhi but the use of that strain was discontinued. Germanier (Germanier, R. and Furer, E., 1975, J. Infect. Dis. 131:553; Germanier, R., 1984 in Bacterial Vaccines, Academic Press, New York, pp. 137–165) advocated the use of a galE mutant of *S. typhi* as an oral vaccine against human typhoid fever.

The *S. typhi* galE mutant, Ty21a, is capable of eliciting a protective response against the pathogenic parental strain in human volunteers (Levine, M. M., et al., 1983, Microbiol. Rev. 47:510; Wahdan, M. H., et al., 1982, J. Infect. Dis. 145:292). The Ty21a strain also has yielded promising protective results in a field test with 28,000 school children in Alexandria, Egypt (Wahdan, M. H., et al., 1982, J. Infect. Dis. 145:292). This vaccine strain is being marketed in Europe as a typhoid fever vaccine. However, a major disadvantage of this vaccine strain is that it exhibits variable viability due to killing by exogenous galactose. Addition of galactose has two effects on the Ty21a (galE mutant) strain. Firstly, it results in the accumulation of toxic galactose-1-phosphate, causing reduced viability. Secondly, added galactose is incorporated into the polysaccharide chain of lipopolysaccharide, which is necessary for immunogenicity. These opposing requirements result in variability in viability and in immunogenicity of the vaccine strain.

Recently, Stocker and his coworkers have described a reliable method to achieve attenuation of Salmonella (e.g., Hoiseth and Stocker, 1981, Nature 291:238; Stocker et al., 1982, Develop. Biol. Standard 53:47; and U.S. Pat. No. 4,550,081). In this method, specific deletion mutations affecting the aromatic biosynthetic pathway are introduced by transduction. Specifically, deletions of the gene aroA result in pleiotropic requirements for phenylalanine, tryptophan, tyrosine, and the folic acid precursor, p-aminobenzoic acid, and the enterochelin precursor, dihydroxybenzoic acid. The aromatic amino acids are present in animal tissues, but p-aminobenzoic acid is absent; folic acid which may be present in animal cells is not assimilated by members of the Enterobacteriaceae. In addition, absence of enterochelin results in the requirement for iron in aroA Salmonella strains.

Since the introduction of techniques for the precise attenuation of Salmonella, a number of vaccination studies have been undertaken in animal model systems (Lindberg, A. A. and Robertsson, J. A., 1983, Infect. Immun. 41:751; Robertsson, J. A., et al., 1983, Infect. Immun. 41:742; Smith, B. P., et al., 1984, Am. J. Vet. Res. 45:2231; Smith, B. P., et al., 1984, Am. J. Vet. Res. 45:59; Stocker, B. A. D., et al., 1982, Develop. Biol. Standard 53:47). Using an aroA derivative of S. typhimurium UCD 108-11, SL1479, in the calf model system, Lindberg and coworkers demonstrated invasiveness of SL1479, and showed that calves vaccinated with SL1479 cleared the vaccine organism quickly from the gut and tissues Oral vaccination with live SL1479 gave greater protection and cell-mediated immune reactivity against S. typhimurium UCD 108-11 infection than that obtained by intraperitoneal vaccination with heat-killed organisms. In other studies, Smith and colleagues demonstrated protection in calves by vaccination with SL1479, against challenge by the virulent parental strain.

A number of groups have demonstrated expression of heterologous genes in Salmonella. Formal and colleagues transferred the genes for the form I antigen of *Shigella sonnei* into *Salmonella typhi* Ty21a (see e.g., Formal, S. B., et al., 1981, Infect. Immun. 34:746; Tramont et al., 1984, J. Inf. Dis. 149:133; and U.S. Pat. No. 4,632,830, by Formal et al.). The form I antigen of Shigella is associated with immunity elicited by vaccination with live attenuated *Shigella sonnei*. Subcutaneous or intraperitoneal injection of living transconjugants protected mice against intraperitoneal challenge by either *S. typhi* Ty21a or *Shigella sonnei*. Also, by conjugation experiments, Yamamoto and coworkers (Yamamoto, T., et al., 1982, J. Bacteriol. 150:1482) introduced an *E. coli* colonization factor antigen (CFA/I) into *Salmonella typhi* Ty21a and demonstrated expression of the antigen.

Clements et al. (1983, Infect. Immun. 53:685) tested antibody responses to the B subunit of heat labile toxin (LT-B), derived from enteroinvasive *E. coli* (EIEC) of human origin (Strain H10407), expressed on a recombinant plasmid in *S. typhi* Ty21a. Since *S. typhi* has a host range limited to humans and higher primates, immunogenicity was tested in an animal model. Intraperitoneal injection of the heterologous LT-B in Ty21a resulted in serum antibody responses to LT-B in mice (Clements, J. D. and S. El-Morshidy, 1984, Infect. Immun. 46:564). Subsequently, LT-B producing recombinants were examined in an attenuated Salmonella strain infectious for mice (Clements, J. D., et al., 1986, Infect. Immun. 53:685). In this study, the LT-B gene was introduced into an aroA attenuated *S. enteriditis* serotype dublin strain: SL1438. The parental *S. enteriditis dublin* strain is virulent in BALB/c mice. After oral vaccination with the recombinant Salmonella strain, EL23, a significant increase in mucosal anti-LT-B IgA was observed. The response to LT-B produced by the recombinant organism was less marked than the immune response to purified LT-B injected intraperitoneally or given orally (id.).

In another study, after introduction of LT-B-encoding plasmids into a aroA deletion mutant of *S. typhimurium*, SL3261, oral or intraperitoneal vaccination with the recombinant bacteria in mice induced an antibody response to LT-B (Maskell et al., 1987, Microbial Pathogenesis 2:211).

Several other groups have also reported expression of heterologous antigens in attenuated Salmonella. Manning and coworkers (Manning, P. A., et al., 1986, Infect. Immun. 53:272) have cloned the gene clusters responsible for lipopolysaccharide synthesis of the O antigens of the major biotypes of *Vibrio cholerae*: Inaba and Ogawa. These gene clusters were expressed on the surface of *S. typhi* Ty21a. The K88 fimbrial adhesin antigen of *Escherichia coli* strains associated with diarrhea of neonatal piglets has been cloned and expressed in *S. typhimurium* SL3261 (Dougan et al., 1986, Infect. Immun. 52:344). Antibodies against the K88 antigen were obtained from sera of mice receiving either oral or intravenous doses of the recombinant *S. typhimurium*. In addition, beta-galactosidase has been expressed in *S. typhimurium* SL3261, and specific anti-beta-galactosidase antibodies were elicited by administration of the recombinant bacteria to mice (Brown, A., et al., 1987, J. Inf. Dis. 155:86), demonstrating that the intracellular beta-galactosidase protein can also provoke an immune response.

3. SUMMARY OF THE INVENTION

The present invention is directed to attenuated strains of enteroinvasive bacteria that express peptides and proteins related to epitopes of the malaria parasites of the genus Plasmodium. The bacterial strains of the invention which can multiply in a host without causing

significant disease or disorder. and which express Plasmodium-related peptides that induce a protective immune response against malaria. can be used in live vaccine formulations for malaria. Such vaccine formulations can be univalent or multivalent.

The expression of Plasmodium epitopes in attenuated enteroinvasive bacteria in the vaccine formulations of the invention provides protective immunity against malaria due to the ability to evoke a cell-mediated immune response in addition to a humoral response. Cell-mediated immunity directed against the Plasmodium epitope results from the invasive properties of the bacteria. which allow presentation of the epitope to the immune system in a manner which can induce cell-mediated immunity.

In particular. the vaccine vector strains of the present invention comprise attenuated Salmonella bacteria which retain their enteroinvasive properties but lose in large part their virulence properties. By obtaining expression of a malarial epitope in attenuated Salmonella, the epitope can be effectively presented to cells important in immune recognition by bacterial invasion. without bacterial persistence or virulence. Thus. effective vaccines against malaria can be achieved. In a preferred embodiment of the invention. a gene or gene fragment encoding all or part of the circumsporozoite malaria antigen can be expressed in Salmonella bacteria that have been attenuated by chromosomal deletion of gene(s) for aromatic compound biosynthesis. for use as a live vaccine for malaria.

The present invention also relates to the methods for expression of the malaria proteins or fragments thereof within attenuated enteroinvasive bacteria. The invention demonstrates the use of plasmid vectors designed for malaria peptide or protein expression in attenuated enteroinvasive bacteria. In particular embodiments. the invention is directed to methods of obtaining expression of circumsporozoite proteins in attenuated Salmonella spp.. and relates to DNA sequences encoding the circumsporozoite proteins of *P. falciparum. P. vivax. P. ovale. P. malariae. P. berghei. P. yoelii. P. knowlesi.* and *P. cynomolgi.* The circumsporozoite proteins of the above species of Plasmodium can be expressed in an attenuated Salmonella strain which is enteroinvasive in the animal host for the appropriate malaria parasite.

In another embodiment. the invention relates to the expression. by attenuated enteroinvasive bacteria. of malaria proteins as recombinant fusion proteins. For example. DNA encoding the circumsporozoite protein or an epitope thereof can be joined to the gene for the B subunit of the heat-labile enterotoxin of *E. coli* (LT-B), or a portion thereof, in order to achieve enhanced immunogenicity.

In specific embodiments of the present invention described in the examples sections herein, the construction of recombinant plasmid expression vectors which encode epitopes of the circumsporozoite protein of *P. berghei.* or of *P. falciparum.* are described. The expression of recombinant LT-B/CS fusion proteins in attenuated Salmonella strains is demonstrated. The recombinant Salmonella which express CS peptides are shown to elicit anti-CS antibody production in mice, and to provoke immune responses which protect against malaria infection upon sporozoite challenge.

3.1. DEFINITIONS

CS =	circumsporozoite
CSP reaction =	circumsporozoite precipitin reaction
DNase =	deoxyribonuclease
DTT =	dithiothreitol
EIEC =	enteroinvasive *E. coli*
ELISA =	enzyme-linked immunoabsorbent assay
i.p. =	intraperitoneally
IPTG =	isopropylthio-beta-D-galactoside
ISI =	inhibition of sporozoite invasion
kD =	kiloDalton
KLH =	keyhole limpet hemocyanin
LB =	Luria broth
LT-B =	the B subunit of the heat-labile enterotoxin of *E. coli*
mAb =	monoclonal antibody
PAGE =	polyacrylamide gel electrophoresis
PBS =	phosphate-buffered saline
P_L =	leftward promoter of bacteriophage lambda
P_R =	rightward promoter of bacteriophage lambda.
RNase =	ribonuclease
S/D =	Shine-Dalgarno
SDS =	sodium dodecyl sulfate

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Nucleotide sequence of the *P. berghei* circumsporozoite gene. Sequence data is according to Weber et al.. 1987. Experimental Parasitol. 63:295. The gene sequence comprises approximately 75% of the sequence of the mature gene and includes the sequence of linkers used in the original cloning of the gene in lambda gt11. Major restriction enzyme sites are indicated. The *P. berghei* specific DNA starts with the CGA codon encompassing the first NruI site.

FIG. 2. Nucleotide and amino acid sequence of the *P. berghei* circumsporozoite protein. The repeated immunodominant epitopes are shown in brackets. and consist of a consensus octapeptide repeat. DPAPPNAN. and a second less frequent octapeptide repeat, DPPPPNPN. Regions I and II are underlined. Repeated dipeptide units are also shown in brackets.

FIG. 3. Gene and protein sequence of LT-B derived from *Escherichia coli* H10407. The DNA sequence and protein sequence of the coding region of the LT-B gene is included within a 588 base pair EcoRI-HindIII restriction fragment. A short Shine-Dalgarno site is within seven base pairs of the initiating codon ATG, and is underlined. The protein sequence of LT-B includes a 21 amino acid signal sequence (shown in brackets) which is processed in the mature form, beginning with alanine. The ClaI, XmaI, and SpeI restriction enzyme recognition sites in LT-B which were useful in constructing fusion protein molecules are shown.

FIG. 4. Is a diagrammatic representation of the construction of plasmid pPX100, a vector which expresses LT-B under the control of the lac operon. Plasmid pJC217 was digested with EcoRI and religated to delete 180 base pairs of DNA including extraneous restriction sites of the polylinker, to yield plasmid pPX100.

FIG. 5. Is a diagrammatic representation of the construction of plasmid vectors which express LT-B/CS fusion proteins under the control of the lac operon. The DNA sequence of the *P. berghei* CS gene shown in FIG. 1 encompasses a large 1.1 kilobase pair NruI and a smaller 670 base pair XmnI fragment which were both

inserted into the filled out ClaI site of pPX100. to yield plasmids pPX1515 and pPX1520.

FIG. 6. Is a diagrammatic representation of portions of vectors constructed to express LT-B/CS fusion proteins regulated by the translation initiation signals of LT-B. The 670 base pair XmnI CS fragment described in FIG. 5 was inserted at the ClaI site (pPX1515). at the XmaI (pPX1523) or the SpeI (pPX1525) site of the LT-B sequence. The resulting vectors express LT-B/CS fusion proteins using the translation initiation signals of the LT-B protein. Relative positions of translation stop codons within the sequence derived from the LT-B insert are indicated by asterisks.

FIG. 7. Is a diagrammatic representation of the construction of tac promoter-driven LT-B/CS fusion proteins The EcoRI-HindIII restriction enzyme fragments containing the LT-B/CS fusion sequence. from either pPX1515, pPX1523. or pPX1525. were isolated and ligated into the EcoRI-HindIII sites of pKK223. The resulting plasmids can express the LT-B/CS fusion proteins under the control of the tac promoter. The construction of pPX1528 is shown as an example.

FIG. 8. Is a diagrammatic representation of the construction of PL promoter-driven LT-B/CS fusion proteins. The EcoRI-HindIII fragments. containing the LT-B/CS fusion sequences. isolated from either pPX1515. pPX1523. or pPX1525. were "filled out" with Klenow enzyme and ligated into the HpaI site of plasmid pP_L lambda. The resulting plasmids can express LT-B/CS fusion proteins under the control of the P_L promoter. The construction of pPX1601 is shown as an example.

FIG. 9. Is a diagrammatic representation of the construction of P_L promoter-driven expression vector pPX1600. An oligonucleotide encoding several restriction enzyme sites. a consensus Shine-Dalgarno sequence. a translation initiation codon. and translation termination codons in all three reading frames was synthesized and ligated into the HpaI site of pP_L lambda to yield pPX1600. pPX1600 can be used to conveniently insert and express heterologous sequences under the control of the P_L promoter.

FIG. 10. Is a diagrammatic representation of the construction of a plasmid vector containing the *P. berghei* CS gene driven by the P_L promoter. The 670 base pair XmnI fragment of the *P. berghei* CS gene was isolated and ligated directly into the filled out NcoI site of plasmid pPX1600 to yield pPX1529. Plasmid pPX1529 can express the *P. berghei* CS gene sequence under the control of the P_L promoter.

FIG. 11. Is a diagrammatic representation of the construction of plasmid vectors which encode immunodominant epitopes of *P. falciparum* or *P. berghei* CS protein, driven by the P_L promoter. By ligating polymerized oligonucleotides encoding CS epitopes (obtained as described in Section 7.7, infra), into the filled out NcoI site of pPX1600, various plasmids containing the oligonucleotides as inserts were isolated. For purposes of demonstration, the insertion of an oligonucleotide encoding four repeats of the *P. falciparum* CS epitope is shown. A monomeric insert results from the insertion of one copy of the oligonucleotide into the NcoI site. The resulting plasmids can express CS epitopes under the control of the P_L promoter.

FIG. 12. Is a diagrammatic representation of the construction of a plasmid vector which can express the full length *P. falciparum* CS protein gene under the control of the P_L promoter. The StuI-RsaI DNA fragment of the *P. falciparum* CS protein gene was ligated into the StuI site of plasmid pPX1600 to yield plasmid pPX1534. which expresses the full length CS gene (lacking only a region encoding the putative 16 amino acid signal sequence) from the P_L promoter.

FIG. 13. Expression of LT-B/CS fusion protein in *S. dublin* SL1438 with increasing strength of promoter. The gene encoding a fusion protein in which 30 amino acids of mature LT-B is fused in-frame with 223 amino acids of the *P. berghei* CS protein, was linked. using the same translation initiation signals. to different promoters. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for detection of CS protein with anti-CS mAb 3.28. in a western blotting procedure. The following vectors were expressed in *S. dublin* SL1438: Lane 1, vector pUC8; Lane 2, pPX1515 (lac promoter); Lane 3, pPX1528 (tac promoter); Lane 4, pPX1601 (P_L promoter). The arrow indicates the position of the CS protein.

FIG. 14. Expression in Salmonella dublin of betagalactosidase/CS or LT-B/CS fusion proteins driven by the lac promoter. Proteins synthesized in *S. dublin* SL1438 were separated by SDS-polyacrylamide gel electrophoresis. and transferred to nitrocellulose for detection of the CS-specific epitope with anti-CS mAb 3.28. in a western blotting procedure. The following vectors were expressed in *S. dublin* SL1438: Lane 1, vector pUC8; Lane 2, pPX1512; Lane 3. pPX1514; Lane 4. pPX1515; Lane 5, pPX1520; Lane 6. pPX1522. (For a description of each of the plasmid constructions, see Sections 7.1–7.9, infra).

FIG. 15. Expression of LT-B/CS *P. berghei* fusion protein, under the control of various promoters, in *S. typhimurium. S. dublin.* and *S. typhi.* aroA mutants of the indicated Salmonella strains were grown to midlog phase in Luria broth containing 50 micrograms per ml of ampicillin. Total protein samples were subjected to electrophoresis in SDS-PAGE and western blotting as described in Sections 6.9 and 6.10. The arrows on the left indicate protein molecular weights expressed in kiloDaltons; the arrow on the right indicates the position of the LT-B/CS fusion protein.

FIG. 16. Isoelectric focussing of proteins obtained from *E. coli* and Salmonella strains which express the *P. falciparum* repeat epitope fused to the first thirty amino acids of LT-B. Sonicated extracts of the indicated strains, containing approximately 50 micrograms of protein, were subjected to isoelectric focussing in a vertical gel apparatus as described in Section 6.10. Ty523 is an *S. typhi* aroA mutant; SL3261 is an *S. typhimurium* aroA mutant, and SL1438 is an *S. dublin* aroA mutant. *E. coli* strain JM103 containing plasmid pPX1532 was induced with 1 mM IPTG; as a control, the same strain without IPTG induction is shown. The principal immunoreactive species are indicated by arrows at the right.

FIG. 17. Anti-CS protein serum antibody response to recombinant Salmonella dublin SL1438 expressing an LT-B/CS fusion protein, or LT-B. C57bl/6 mice received a primary vaccinating dose of 10^7 *S. dublin* carrying the indicated plasmids by the intraperitoneal (i.p.) route. Mice were boosted i.p. at week 4 with organisms of the same strain as the primary dose. Anti-CS protein antibody response was measured in an ELISA with a 1:160 dilution of serum taken at week four. Data are expressed as OD (optical density) values measured at 410 nm. pPX1527 encodes LT-B driven by the tac pro-

moter; pPX1528 encodes an LT-B/CS fusion protein driven by the tac promoter; and pPX1601 encodes an LT-B/CS fusion protein driven by the P_L promoter.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to attenuated strains of enteroinvasive bacteria that express peptides and proteins related to epitopes of the malaria parasites of the genus Plasmodium. The bacterial strains of the present invention which can multiply in a host without causing significant disease or disorder, and which express Plasmodium-related peptides that induce a protective immune response against malaria, can be used in live vaccine formulations for malaria. Such vaccine formulations can be univalent or multivalent.

The expression of Plasmodium epitopes in the vaccine formulations of the present invention provides protective immunity against malaria due to the ability of such vaccine formulations to evoke a cell-mediated immune response in addition to a humoral response. Cell-mediated immunity directed against the Plasmodium epitope results from the invasive properties of the bacteria, which allow presentation of the epitope to the host immune system in a manner which can induce cell-mediated immunity.

In particular, the vaccine vector strains of the present invention comprise attenuated Salmonella bacteria which retain their enteroinvasive properties but lose in large part their virulence properties. Bacteria of the genus Salmonella can invade intestinal epithelial cells and establish systemic infections by invading the reticuloendothelial system of the host animal. By causing attenuation of virulent bacteria, invasive properties of the bacteria are retained but their virulence properties are lost.

In a preferred embodiment of the invention, a gene or gene fragment encoding all or part of the circumsporozoite malaria antigen can be expressed in Salmonella bacteria that have been attenuated by chromosomal deletion of gene(s) for aromatic compound biosynthesis, for use as a live vaccine for malaria.

By obtaining expression of the circumsporozoite proteins of the malarial sporozoites in attenuated Salmonella, sporozoite antigens can be effectively delivered to the specific cells important in immune recognition by bacterial invasion without bacterial persistence or virulence. In this fashion, effective vaccines against malaria sporozoites can be achieved.

The present invention also relates to the methods for expression of the malaria proteins or fragments thereof within attenuated enteroinvasive bacteria. The invention also demonstrates the use of plasmid vectors designed for malaria peptide or protein expression in attenuated enteroinvasive bacteria. In particular embodiments, this invention is directed to methods of obtaining expression of circumsporozoite proteins in attenuated Salmonella spp. and relates to DNA sequences encoding the circumsporozoite proteins of *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. berghei*, *P. yoelii*, *P. knowlesi*, and *P. cynomolgi*. The circumsporozoite proteins of the above species of Plasmodium can be expressed in an attenuated Salmonella strain which is enteroinvasive in the animal host for the appropriate malaria parasite. In a specific embodiment, the invention also relates to the expression of the *P. falciparum* circumsporozoite protein or immunogenic portion thereof in attenuated *S. typhi* containing aroA chromosomal deletion mutation(s), for use as a live vaccine against the most serious form of human malaria. In another specific embodiment, the invention relates to the expression of *P. berghei* circumsporozoite protein in attenuated Salmonella which are enteroinvasive for mice, as a model system to study the efficacy of the live vaccines of the present invention for human malaria.

In another embodiment, the invention relates to the expression of malaria proteins as recombinant fusion proteins. Such fusion proteins can exhibit increased immunogenicity of malaria epitopes. In a particular embodiment, by obtaining fusion circumsporozoite proteins using recombinant DNA technology, the immunogenicity of the circumsporozoite protein can be modified. In such an embodiment, DNA encoding the circumsporozoite protein or an epitope thereof can be joined to the gene for the B-subunit of the labile enterotoxin of *E. coli* (LT-B), or a portion thereof, in order to achieve modified immunogenicity. Fusion LT-B/circumsporozoite protein immunogens expressed in Salmonella spp. can supply additional T cell helper functions. In this embodiment, coupling of an epitope of the circumsporozoite protein to LT-B also directs the recombinant DNA-derived fusion protein to the periplasmic space of the invasive Salmonella, and aids in antigen presentation.

The method of the invention may be divided into the following stages solely for the purpose of description: (a) isolation of a gene, or gene fragment, encoding an epitope of a malaria parasite; (b) insertion of the gene or gene fragment into an expression vector; (c) transfer to and expression of the gene or gene fragment in an attenuated enteroinvasive bacteria; (d) determination of immunopotency of the malaria epitope expressed by the recombinant enteroinvasive bacteria; and (e) formulation of a vaccine.

In specific embodiments of the present invention described in the examples sections herein, we describe the construction of recombinant plasmid expression vectors encoding epitopes of the circumsporozoite protein of *P. berghei*, or of *P. falciparum*. We further describe the expression of recombinant LT-B/CS fusion proteins in attenuated Salmonella strains. The recombinant Salmonella which express CS peptides are shown to elicit anti-CS antibody production in mice, and to provoke immune responses which protect against malaria infection upon sporozoite challenge.

5.1. ISOLATION OF GENE OR GENE FRAGMENTS ENCODING PLASMODIUM EPITOPES

Any DNA sequence which encodes a Plasmodium epitope, which when expressed as a fusion or nonfusion protein in an attenuated enteroinvasive bacteria, produces protective immunity against malaria, can be isolated for use in the vaccine formulations of the present invention. The species of Plasmodium which can serve as DNA sources include but are not limited to the human malaria parasites *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and the animal malaria parasites *P. berghei*, *P. yoelii*, *P. knowlesi*, and *P. cynomolgi*.

There are numerous genes which encode a Plasmodium antigen that can serve as a source of the DNA sequence to be isolated and expressed in attenuated enteroinvasive bacteria according to the present invention. The antigens, or fragments thereof, which can be expressed by recombinant bacteria in the vaccine formulations of the invention are antigens which are ex-

pressed by the malaria parasite at any of the various stages in its life cycle, such as the sporozoite, exoerythrocytic (development in hepatic parenchymal cells), asexual erythrocytic, or sexual (e.g., gametes, zygotes, ookinetes) stages. The antigen can be expressed by the malaria parasite itself or by an infected cell. The Plasmodium antigens which may be used include but are not limited to those described in the following publications, incorporated by reference herein:

Vaccines85, 1985, Lerner, R. A., et al., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 1-57

Vaccines86, 1986, Brown, F., et al., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp. 135-179

Vaccines87, 1987, Channock et al., eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 81-106, 117-124

Kemp, D. J., et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:3787

Anders, R. F., et al., 1984, Mol. Biol. Med. 2(3):177-191

Miller, L. H., et al., 1984, J. Immunol. 132(1):438-442

Carter, R., et al., Nov. 13, 1984, Philos. Trans. R. Soc. Lond. (Biol.) 307(1131):201-213

Holder, A. A. and Freeman, R. R., 1981, Nature 294:361

Leech, J. H., et al., 1984, J. Exp. Med. 159:1567

Rener, J., et al., 1983, J. Exp. Med. 158:971

Dame, J. B., et al., 1984, Science 225:593

Arnot, D. E., et al., 1985, Science 230:815

Coppel, R. L., et al., 1983, Nature 306:751

Coppel, R. L., et al., 1984, Nature 310:789

Holder, A. A., et al., 1985, Nature 317:270

Ardeshir, F., et al., 1987, EMBO J. 6:493

Ravetch, J. V., et al., 1985, Science 227:1593

Stahl, H.-D., et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:543

Langsley, G., et al., 1985, Nucl. Acids Res. 11:4191

Coppel, R. L., et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:5121

Howard, R. J., et al., 1987, J. Cell Biol. 104:1269

Buranakitjaroen, P. and Newbold, C. I., 1987, Mol. Biochem. Parasitol. 22:65

Schofield, L., et al., 1986, Mol. Biochem. Parasitol. 18:183

Knowles, G. and Davidson, W. L., 1984, Am. J. Trop. Med. Hyg. 33:789

Kilejian, A., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:4650

Leech, J. H., et al., 1984, J. Cell. Biol. 98:1256

Hadley, T. J., et al., 1986, Ann. Rev. Microbiol. 40:451

Camus, D. and Hadley, T. J., 1985, Science 230:553

Vermeulen, A. M., et al., 1985, J. Exp. Med. 162:1460.

Vermeulen, A. M., et al., 1986, Mol. Biochem. Parasitol. 20:155

Kumar, N. and Carter, R., 1984, Mol. Biochem. Parasitol. 13:333

Patarroyo, M. E., et al., 1987, Nature 328:629-632

Miller, L. H., et al., 1984, Phil. Trans. R. Soc. Lond. B307:99-115.

As particular examples, such antigens include the circumsporozoite antigen; the *P. falciparum* blood-stage ring-infected erythrocyte surface antigen (RESA), S antigen, Falciparum interspersed repeat antigen (FIRA), glycophorin binding protein (GBP), Pf 195 kD antigen, circumsporozoite protein-related antigen (CRA), Pf 155 antigen, Pf 75 kD antigen, Pf EMP 2 antigen, and Pf knob-associated antigens; *P. falciparum* sexual stage antigens of 260,000, 59,000 and 53,000 molecular weight, antigens of 230,000, 48,000, and 45,000 molecular weight, etc.

In a particular embodiment, a Plasmodium peptide can be expressed as a fusion protein with a secreted protein sequence of a bacteria, so that the recombinant fusion protein is directed to the periplasmic space of the bacteria, thus aiding presentation to the immune system and enhancing immunogenicity.

Although extracellular localization of the Plasmodium epitope expressed by the recombinant enteroinvasive bacteria is preferred, extracellular localization is not required, since intracellular localization can also evoke an effective immune response. When beta-galactosidase, an intracellular protein, was expressed in *S. typhimurium* SL3261, specific anti-beta-galactosidase antibodies were elicited by administration of the recombinant bacteria to mice (Brown, A., et al., 1987, J. Inf. Disc. 155:86).

In a preferred embodiment, the malaria epitope to be expressed is an epitope of the circumsporozoite (CS) protein of a species of Plasmodium. Analogous CS proteins have been identified on the surfaces of sporozoites of all serotypes of Plasmodium. Circumsporozoite protein antigens expressed in attenuated Salmonella spp. can be used as live vaccines directed against sporozoites, the invasive form of malaria parasites transmitted by the female Anopheles mosquito. The genes which encode a CS epitope and which those Plasmodium species listed supra. In particular, those genes encoding the CS proteins of the human malaria parasites *P. falciparum*, *P. vivax*, the simian parasites *P. cynomolgi* and *P. knowlesi*, and the rodent parasite *P. berghei* can be used; these genes have been cloned and sequenced, as reported in the following publications, which are incorporated by reference herein: Dame, J. B., et al., 1984, Science 225:593; Arnot, D. D., et al., 1985, Science 230:815; Weber et al., 1987, Exp. Parasitol. 63:295; Enea, V., et al., 1984, Science 225:628; Enea, V., et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:7520; Godson, G. N., et al., 1983, Nature 305:29; and McCutchan, T. F., et al., 1985, Science 230:1381. A characteristic feature of the CS genes of each of the parasites is a central region which comprises over one third of the protein and contains a large series of repeated peptide sequences (Dame, J. B., et al., 1984, Science 225:593; Ozaki, L. S., et al., 1983, Cell 34:815). The primary amino acid sequence, the length of the repeating sequence, and the number of repeats varies with each species of parasite. As examples, the gene encoding the CS protein of *P. falciparum* specifies a central repeat region of a tetrapeptide (asn-ala-asn-pro) repeated thirty-seven times, interrupted in four locations by a variant tetrapeptide (asn-val-asp-pro). The central repeat region of *P. vivax* contains nineteen nonapeptides; the central sequence of *P. knowlesi* contains eight dodecapeptides, and the repeat region of *P. berghei* contains twelve octapeptides. Comparison of sequences from *P. knowlesi* (H strain) and *P. falciparum* and *P. vivax* revealed no sequence homology, except for two short amino acid sequences flanking the repeat region, termed Region I and Region II. The repeat regions appear to be highly conserved within the human malaria parasites *P. falciparum* and *P. vivax* (Weber, J. L. and Hockmeyer, W. T., 1984, Mol. Biochem. Parasitol. 15:305; Zavala, F., et al., 1985, J. Immunol. 135:2750), though intra-species variation has been observed in *P. knowlesi* and *P. cynomolgi*. The repeat region also appears to be immunodominant

(Dame, J. B., et al., 1984. Science 225:593; Hockmeyer, W. T. and Dame, J. B., 1985. in Immunobiology of Proteins and Peptides III. Atassi, M. Z., ed., Plenum Press, New York, pp. 233–246; Zavala, F., et al., 1983. J. Exp. Med. 157:1947; Zavala, F., et al., 1985. Science 228:1436). In preferred embodiments of the invention, DNA sequences containing the repeat region, Region I, or Region II, can be isolated for use in the vaccine formulations of the present invention. For example, in one embodiment, the peptide asn-ala-asn-pro, related to the *P. falciparum* CS repeat region, can be expressed by the recombinant bacteria of the invention. In another embodiment, the peptide asp-pro-ala-pro-pro-asn-ala-asn, representing the *P. berghei* CS protein repeat region, can be expressed.

The Plasmodium CS peptides to be expressed in recombinant enteroinvasive bacteria according to the present invention, whether produced by recombinant DNA methods, chemical synthesis, or purification techniques, include but are not limited to all or part of the amino acid sequences of Plasmodium-specific antigens, including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine, and histidine. The negatively charged (acidic) amino acids include aspartic and glutamic acid.

A DNA sequence encoding a malarial epitope which is a hapten, i.e., a molecule that is antigenic in that it can react selectively with cognate antibodies, but not immunogenic in that it cannot elicit an immune response, can be isolated for use in the vaccine formulations of the present invention, since it is envisioned that, in particular embodiments, presentation by the enteroinvasive attenuated bacteria of the invention can confer immunogenicity to the hapten expressed by the bacteria. As particular examples, expression of a malarial hapten as a fusion protein with an immunogenic peptide, e.g., derived from *E. coli* enterotoxin subunit B (LT-B), can confer immunogenicity.

5.2. CONSTRUCTION OF EXPRESSION VECTORS CONTAINING SEQUENCES WHICH ENCODE A PLASMODIUM EPITOPE

In this aspect of the invention, the desired DNA sequence encoding the malarial epitope is inserted, using recombinant DNA methodology (see Maniatis, T., 1982. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), into an expression vector so that it can be expressed under the control of an active promoter in the host attenuated enteroinvasive bacteria. The DNA sequence encoding the Plasmodium epitope can be obtained from any of numerous sources such as cloned malarial DNA, genomic malarial DNA, cDNA of malarial RNA, or chemically synthesized DNA.

In order to generate Plasmodium DNA fragments, the Plasmodium DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNaseI in the presence of manganese, or mung bean nuclease (McCutchan et al., 1984. Science 225:626), to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including, but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Any restriction enzyme or combination of restriction enzymes may be used to generate Plasmodium DNA fragment(s) containing the desired epitope(s), provided the enzymes do not destroy the immunopotency of the encoded product. Consequently, many restriction enzyme combinations may be used to generate DNA fragments which, when inserted into a appropriate vector, are capable of directing the production of the peptide containing the Plasmodium epitope.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired malaria sequence may be accomplished in a number of ways. For example, if a small amount of the desired DNA sequence or a homologous sequence is previously available, it can be used as a labeled probe (e.g., nick translated) to detect the DNA fragment containing the desired sequence, by nucleic acid hybridization. Alternatively, if the sequence of the derived gene or gene fragment is known, isolated fragments or portions thereof can be sequenced by methods known in the art, and identified by a comparison of the derived sequence to that of the known DNA or protein sequence. Alternatively, the desired fragment can be identified by techniques including but not limited to mRNA selection, making cDNA to the identified mRNA, chemically synthesizing the gene sequence (provided the sequence is known), or selection on the basis of expression of the encoded protein (e.g., by antibody binding) after "shotgun cloning" of various DNA fragments into an expression system.

Once identified and isolated, the Plasmodium DNA fragment containing the sequence(s) of interest is then inserted into a vector which is capable of replication and expression in the host enteroinvasive bacteria. The Plasmodium DNA may be inserted into the bacterial chromosomal DNA. Alternatively, in a preferred embodiment, the Plasmodium DNA is inserted into a cloning vector which can exist episomally, e.g., a plasmid or bacteriophage, which is then used to transform or infect appropriate host bacterial cells, where the Plasmodium DNA is replicated and expressed.

If the complementary restriction sites used to fragment the Plasmodium DNA are not present in the cloning vector, the ends of the DNA molecules may be modified (for example, see Section 6, infra). Such modifications include producing blunt ends by digesting back single-stranded DNA termini or by filling the single-stranded termini so that the ends can be blunt-end ligated. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction site recognition sequences. According to other methods, the cleaved vector and the Plasmodium DNA fragment may be modified by homopolymeric tailing.

The transformation of attenuated enteroinvasive bacteria with the recombinant DNA molecules that incorporate the Plasmodium DNA enables generation of multiple copies of the Plasmodium sequence. A variety of vector systems may be utilized for expression within the bacterial host, including but not limited to plasmids such as pUC plasmids and derivatives, PBR322 plasmid and derivatives, bacteriophage such as lambda and its derivatives, and cosmids. In a specific embodiment, plasmid cloning vectors which can be used include derivatives of ColE1 type replicons (for additional information, see Oka et al., 1979, Mol. Gen. Genet. 172:151–159). The ColE1 plasmids are stably maintained in *E. coli* and *Salmonella typhimurium* strains as monomeric molecules with a copy number of about 15–20 copies per cell. Various regulatory expression elements can be used, which are any of a number of suitable transcription and translation elements that are active in the attenuated enteroinvasive bacteria of the invention. For instance, promoters which may be used to direct the expression of the Plasmodium DNA sequence include but are not limited to the lactose operon promoter of *E. coli*, the hybrid trp-lac UV-5 promoter (tac) (DeBoer, H., et al., 1982, in Promoter Structure and Function, Rodriguez, R. L. and Chamberlain, M. J., eds., Praeger Publishing, New York), the leftward (P_L) and the rightward (P_R) promoters of bacteriophage lambda, the bacteriophage T7 promoter, the trp operon promoter, the lpp promoter (the *E. coli* lipoprotein gene promoter; Nakamura, K. and Inouye, I., 1979, Cell 18:1109–1117), etc. Other promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted sequences.

Specific initiation signals are also required for efficient translation of inserted protein coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire Plasmodium gene including its own initiation codon and adjacent sequences are inserted into the appropriate expression vectors, no additional translational control signals may be needed. However, in cases where only a portion of the gene sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. The initiation codon must furthermore be in phase with the reading frame of the protein coding sequences to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination).

For reviews on maximizing gene expression, see Roberts and Lauer, 1979, Meth. Enzymol. 68:473; and Reznikoff, W. and Gold, M., 1986, Maximizing Gene Expression, Plenum Press, New York.

The Plasmodium peptide or protein can be expressed as a fusion or nonfusion recombinant protein. *E. coli* and other bacteria contain an active proteolytic system which appears to selectively destroy "abnormal" or foreign proteins (Bukhari, A. and Zipser, D., 1973, Nature 243:238). In order to protect eucaryotic proteins expressed in bacteria from proteolytic degradation, one strategy which can be used is to construct hybrid genes in which the foreign sequence is ligated in phase (i.e., in the correct reading frame) with a procaryotic gene. Expression of this hybrid gene results in a fusion protein product (i.e., a protein that is a hybrid of procaryotic and foreign amino acid sequences). In a specific embodiment where the Plasmodium epitope is expressed as part of a fusion protein, the Plasmodium sequence can be fused to a heterologous immunogenic sequence. In a particular embodiment, the Plasmodium sequence can be fused to all or part of the *E. coli* enterotoxin subunit B.

U.S. Pat. No. 4,237,224 to Cohen and Boyer describes production of recombinant plasmids using processes of cleavage with restriction enzymes and joining with DNA ligase by known methods of ligation. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. Because of the general applicability of the techniques described therein, U.S. Pat. No. 4,237,224 is hereby incorporated by reference into the present specification.

Another method for introducing recombinant DNA molecules into unicellular organisms is described by Collins and Hohn in U.S. Pat. No. 4,304,863, which is also incorporated herein by reference. This method utilizes a packaging/transduction system with bacteriophage vectors (cosmids).

5.3. IDENTIFICATION OF RECOMBINANT EXPRESSION VECTORS WHICH REPLICATE AND EXPRESS A PLASMODIUM ANTIGEN OR FRAGMENT THEREOF

Expression vectors containing foreign gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the foreign inserted gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., beta-galactosidase activity, thymidine kinase activity, resistance to antibiotics, etc.) caused by the insertion of foreign genes in the vector. For example, if the Plasmodium DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the Plasmodium insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based on the physical, immunological, or functional properties of the gene product. For example, an ELISA can be used to detect the presence of antigenic determinants which bind the appropriate anti-Plasmodium antibody (e.g., see Section 6.12, infra). Once the desired recombinant molecule is identified and isolated, it can be propagated and prepared in quantity by methods known in the art.

5.4. EXPRESSION BY ATTENUATED ENTEROINVASIVE BACTERIA

The expression vector comprising the malaria DNA sequence should then be transferred into an attenuated

enteroinvasive bacterial cell where it can replicate and be expressed. This can be accomplished by any of numerous methods known in the art including but not limited to transformation (e.g., of isolated plasmid DNA into the attenuated bacterial host), phage transduction, conjugation between bacterial host species, microinjection, etc. In a preferred embodiment involving the use of a plasmid expression vector, the plasmid construction can be isolated and characterized first in *E. coli* K12, before transfer to a Salmonella strain, e.g., by phage transduction (Schmeiger, 1972, Mol. Gen. Genetics 119:75), because of the high transformation frequencies of *E. coli* K12 relative to those of Salmonella such as *S. typhimurium*.

Any of various attenuated enteroinvasive bacteria can be used as a vehicle to express a malaria epitope so that it is effectively presented to the host immune system, in the vaccine formulations of the present invention. The vaccine bacteria retain their invasive properties, but lose in large part their virulence properties, thus allowing them to multiply in the host without causing significant disease or disorder. Examples of enteroinvasive bacteria which, in attenuated forms, may be used in the vaccine formulations of the invention include but are not limited to Salmonella spp., enteroinvasive *E. coli* (EIEC), and Shigella spp. In a preferred embodiment, enteroinvasive bacteria which reside in lymphoid tissues such as the spleen (e.g., Salmonella spp.) are used. Such bacteria can invade gut epithelial tissue, disseminate throughout the reticuloendothelial system, and gain access to mesenteric lymphoid tissue where they multiply and induce humoral and cell-mediated immunity.

Attenuated enteroinvasive bacteria may be obtained by numerous methods including but not limited to chemical mutagenesis, genetic insertion, deletion (Miller, J., 1972, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) or recombination using recombinant DNA methodology (Maniatis, T., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), laboratory selection of natural mutations, etc. Methods for obtaining attenuated Salmonella strains which are non-reverting nonvirulent auxotrophic mutants suitable for use as live vaccines are described in copending U.S. patent application Ser. Nos. 675,381, filed Nov. 27, 1984, and 798,052, filed Nov. 14, 1985, by Stocker, which are incorporated by reference herein in their entirety.

Attenuated Salmonella which can be used in the live vaccine formulations of the invention include but are not limited to those species listed in Table I, infra.

TABLE I

SALMONELLA SPECIES WHICH, IN ATTENUATED FORMS, CAN BE USED IN THE VACCINE FORMULATIONS OF THE PRESENT INVENTION*
S. typhi
(e.g., Ty21a, Ty523, Ty541)
S. typhimurium
(e.g., SL3261, LT241)
S. paratyphi A
S. paratyphi B
S. enteritidis
(e.g., serotype dublin, e.g., strain SL1438)
S. cholerae-suis

*For a complete description of Salmonella serotypes, see Edwards and Ewing, 1986, Classification of the Enterobacteriaceae, 4th Ed., Elsevier, N.Y.

In specific embodiments, Salmonella bacteria that have been attenuated by chromosomal deletion of gene(s) for aromatic compound biosynthesis (aro) or mutation in the galE gene can be used.

S. typhi strains such as Ty523 and Ty541 are avirulent in humans by virtue of attenuation by deletion of the genes encoding aroA and/or purA (Levine, M. M., et al., 1987, J. Clin. Invest. 79:888). Mutants of *S. dublin*, such as SL1438, and of *S. typhimurium*, such as SL3261, can be used in the development of animal model systems, since they are capable of causing animal diseases equivalent to typhoid

5.4.1. ATTENUATION BY galE MUTATIONS

galE mutants can provide a source of attenuated bacteria for use in the vaccine formulations of the present invention. Such galE mutants include but are not limited to the *Salmonella typhi* strains Ty2, Ty21 (Hone et al., 1987, J. Inf. Dis. 156:167), and CDC10-80, and the *Salmonella typhimurium* strains LT-2, LT241, etc. The *S. typhi* galE mutant, Ty21a, and the *S. typhimurium* galE mutant, LT241, are lacking the enzyme UDP-galactose-epimerase and are deficient in two other enzymes of galactose metabolism (Germanier, R. and Furer, E., 1975, J. Infect. Dis. 131:553). LPS (lipopolysaccharide) is synthesized in this strain, but toxic galactose-1-phosphate accumulates and cell lysis ensues.

5.4.2. ATTENUATION BY aro MUTATIONS

aro mutants provide another potential source of attenuated bacteria. Deletions of the gene aroA result in pleiotropic requirements for phenylalanine, tryptophan, tyrosine, and the folic acid precursor, p-aminobenzoic acid, and the enterochelin precursor, dihydroxybenzoic acid. The aromatic amino acids are present in animal tissues, but p-aminobenzoic acid is absent; folic acid which may be present in animal cells is not assimilated by members of the Enterobacteriaceae. In addition, absence of enterochelin results in a requirement for iron in aroA Salmonella vaccine strains (Stocker, B. A. D., et al., 1982, Develop. Biol. Standard 53:47). Thus, deletions in the aroA gene result in biochemical lesions which presumably do not affect other factors which may be important for invasiveness, thus yielding attenuated bacteria which retain invasive properties.

aro mutants which can be used include but are not limited to *S. typhi* strains Ty523 and Ty541, for use in vaccines for humans, and *S. typhimurium* SL3261 and SL1479, and *S. enteriditis* serotype *dublin* SL1438, for use in animals. (See U.S. Pat. No. 4,550,081 for a description of *S. typhimurium* strain SL1479 and *S. dublin* strain SL1438.)

A reliable method to achieve attenuation of Salmonella has been described (Hoiseth, S. K. and Stocker, B. A. D., 1981, Nature 291:238; Stocker, B. A. D., et al., 1982, Develop. Biol. Standard 53:47; and U.S. Pat. No. 4,550,081) and can be used in a particular embodiment of the invention. In this method, specific deletion mutations affecting the aromatic biosynthetic pathway are introduced by transduction. The advantage of this method is that precisely defined mutations can be engineered without the use of chemical or radiation mutagenesis. In principle, a defect in aromatic biosynthesis in Salmonella causes requirements for nutrients not present in adequate free concentrations in animal tissues to support the growth of the bacteria; hence, invading bacteria are attenuated and cannot cause disease. Because mutations are deletions of a large part of one or more genes, mutational reversion is improbable.

5.5. DETERMINATION OF IMMUNOPOTENCY OF THE PLASMODIUM EPITOPE EXPRESSED BY THE RECOMBINANT ENTEROINVASIVE BACTERIA

Immunopotency of the malaria epitope in its live vaccine formulation can be determined by monitoring the immune response of test animals following immunization with the attenuated enteroinvasive bacteria expressing the malaria epitope. Generation of a humoral response may be taken as an indication of a generalized immune response, other components of which, particularly cell-mediated immunity, may be important for protection against the malaria parasite. Test animals may include mice, rabbits, chimpanzees and eventually human subjects. Methods of introduction of the immunogen may include oral, intracerebral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal or any other standard routes of immunizations. The immune response of the test subjects can be analyzed by various approaches such as: the reactivity of the resultant immune serum to malaria antigens, as assayed by known techniques, e.g., enzyme linked immunosorbant assay (ELISA), immunoblots, radioimmunoprecipitations, etc.; or protection from Plasmodium infection and/or attenuation of malaria symptoms in immunized hosts.

5.6. FORMULATION OF A VACCINE

The purpose of this embodiment of the invention is to formulate a vaccine in which the immunogen is an attenuated enteroinvasive bacteria that expresses a malaria epitope so as to elicit a protective immune (humoral and/or cell-mediated) response against Plasmodium infections for the prevention of malaria. The bacteria of the vaccine comprise an attenuated enteroinvasive strain that is infectious for the host to be vaccinated. Such a live vaccine can be univalent or multivalent.

Multivalent vaccines can be prepared from a single or few recombinant attenuated enteroinvasive bacteria which express one or more Plasmodium-related epitopes. The vaccine may also include bacteria that express epitopes of organisms that cause other diseases, in addition to epitopes of malaria parasites. A single enteroinvasive bacteria can express more than one malarial epitope of the same or different antigens, and/or an epitope of a heterologous pathogen. The various epitopes may be expressed within the same protein (i.e., within a fusion protein), on separate proteins coded by the same or different expression vectors, or in different bacteria.

Many methods may be used to introduce the live vaccine formulations of the invention; these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes, including the natural route of infection of the parent wild-type bacterial strain.

In a specific embodiment, attenuated Salmonella expressing an epitope of a malarial circumsporozoite protein can be formulated as a vaccine.

5.6.1 VACCINATION STRATEGIES

Malaria vaccines can be directed at a specific stage in the life cycle of the parasite. In a particular embodiment, the vaccine can be directed at the sporozoite, the stage transmitted by mosquitoes, and which initiates infection in man.

An effective anti-sporozoite vaccine would have the intrinsic advantage of completely preventing infection. After being injected by the mosquito, sporozoites rapidly invade liver cells, where they develop into the stages which infect red blood cells and cause clinical illness. Thus, to completely block sporozoite infection of hepatocytes, high levels of antibodies against the sporozoite should be present.

In another embodiment, the vaccine formulation of the invention can be directed against an erythrocytic stage of the malaria parasite. This vaccine can be especially valuable, since antibodies against the sporozoite stage have no effect on asexual parasite stages which infect erythrocytes, and thus a single sporozoite which escapes the anti-sporozoite antibody-mediated immune response can still initiate a clinical case of malaria. Since mortality from malaria is related to the degree of parasitemia, even a blood-stage vaccine which produced less than complete immunity and reduced the numbers of infected red blood cells would be clinically useful.

Another specific embodiment of the invention involves the concept of transmission blocking immunity. Antibodies against the sexual (gametocyte) stage taken in with the blood meal can block fertilization of the parasite in the midgut of the mosquito, lyse gametes and zygotes, or block development of zygotes. In a particular embodiment, such a vaccine, which offers protection to populations as opposed to individuals, can be used as apart of a multivalent vaccine in malaria control programs.

Previous work with subunit vaccines demonstrated the need for effective malaria vaccine formulations. The CS gene of *P. berghei* has recently been cloned and sequenced (Weber et al., 1987, Experimental Parasitol. 63:295–300; see Section 7.1, infra). The repeat region of *P. berghei* contains four different octapeptides in a total of twelve units, as well as two dipeptides in a sixteen unit repeat. A peptide consisting of two repeats of the consensus octapeptide was coupled to keyhole limpet hemocyanin (KLH). Alternatively, the cloned gene containing approximately 70% of the actual coding sequence of the mature gene product including all of the repeat region was expressed in *E. coli*. The peptide, the recombinant protein, or gamma-irradiated sporozoites were used to immunize mice. Antibody titers measured by ELISA against peptide, the recombinant protein, and intact sporozoites, as well as CSP and ISI activity, were at least as high in the subunit vaccinated groups of mice as in the groups immunized with irradiated sporozoites. Significantly, only the irradiated sporozoite-immunized animals could be protected (90%) against high sporozoite challenge (10^4). Subunit vaccinated animals were protected only at the lowest sporozoite challenge dose (500). Protection was only partial and never exceeded 40% (Egan, J., et al., 1987, Science 236:453).

In recent studies, we have shown that transfer of T cells from sporozoite-immunized mice protected recipient animals, but mice receiving B cells or polyclonal immune sera were not protected, thus suggesting that cellular immunity is important.

The vaccine formulations of the present invention provide protective immunity against malaria, as a result of the expression of malaria epitopes in attenuated enteroinvasive bacteria, which allows the induction of cell-mediated immunity (CMI), in addition to humoral immunity

We speculate that in the normal course of immunization with sporozoites, antibody prevents some but not all the sporozoites from reaching the liver. Sporozoites deposit CS protein on the surface of hepatocytes when they invade and developing exoerythrocytic forms (EE) express epitopes recognized by mAbs raised against sporozoites. These developing EE forms would be likely targets for natural killer cells and cytotoxic T cells or cytokine mediated responses. The failure of parenterally administered subunit CS vaccines to induce protective cellular responses may be a result of inappropriate antigen presentation in association with the MHC (major histocompatibility) molecule, or alternatively could indicate that epitopes critical to sporozoite induced immunity are not present on the CS protein. However, the latter explanation is unlikely, since the CS protein is the only known detectable surface antigen. The likelihood that targeting of the organism to a particular cell type and subsequent presentation of antigen in conjunction with the appropriate MHC molecule may be critical for induction of CMI is supported by the fact that protective immunization against sporozoite challenge requires the intravenous administration of intact, attenuated sporozoites. Neither intramuscular immunization nor use of the freeze-thawed or sonicated organisms induces significant protection (Spitalny, G. L. and Nussenzweig, R. S., 1972, Proc. Helm. Soc. Wash. 39:506).

A solution to this problem is to deliver the expressed CS protein (or other malarial antigen, or epitopes thereof) in an attenuated organism which can facilitate delivery to appropriate antigen-presenting cells, as provided by the vaccine formulations of the present invention. A preferred embodiment of the invention is the use of an avirulent non-pathogenic Salmonella oral vector delivery system. Use of this system can not only preclude some of the potential side effects associated with the use of other delivery vehicles such as vaccinia virus, but can also provide for convenient oral administration of malaria vaccines. This latter point is crucial for developing countries with limited health resources that are unable to support multiple administration of parenterally administered vaccines.

5.6.2. ORAL VACCINATION WITH ATTENUATED SALMONELLA

In a particular embodiment of the present invention, the concept of using attenuated species of Salmonella to deliver foreign antigens is based on the ability of Salmonella spp. to invade gut epithelial tissue and thereby gain access to mesenteric lymphoid tissue. In the mouse typhoid model, Takeuchi (1975, in Microbiology, American Society for Microbiology, Washington, D.C., pp. 174–181) has shown that following the attachment of *S. typhimurium* at the luminal brush border, bacteria invade the villus tip and become engulfed in pinocytized membrane vacuoles. Crossing the distal membrane of epithelial cells, the bacteria can disseminate throughout the reticuloendothelial system. When they reach the lamina propria, Salmonella cells cause an influx of macrophages which ingest the bacteria. Some cells escape phagocytosis and drain into mesenteric lymph nodes where they multiply. We postulate that stimulation of cell mediated responses is a consequence of invasion of the reticuloendothelial system. Thus, an innate feature of the attenuated oral vaccines of the invention is that they be able to invade the intestinal epithelium as can a pathogenic organism, but fail to cause active disease (e.g., because of a precisely defined genetic lesion), resulting in loss of pathogenicity. Oral malaria vaccines based on Salmonella have several advantages over vaccines now being developed. For example, with the appropriate genetic construction, the vector can mimic the sporozoite in surface presentation of the antigen and stimulate both cell-mediated and humoral immunity. Purification steps of a recombinant protein is not necessary, and the live attenuated Salmonella vaccine can be cheaply produced and conveniently administered (e.g., in a lyophilized form). In addition, the probability of adverse reactions based on available animal and human studies is low (Germanier, R., 1984, in Bacterial Vaccines, Academic Press, New York, pp. 137–165; Gilman, R. H., et al., 1977, J. Infect. Dis. 136:717; Levine, M. M., et al., 1983, Microbiol. Rev. 47:510; Smith, B. P., et al., 1984, Am. J. Vet. Res. 45:2231; Smith, B. P., et al., 1984, Am. J. Vet. Res. 45:59; Wray, C., et al., 1982, Develop. Biol. Standard 53:41; Wray, C., et al., 1977, J. Hyg. Camb. 79:17).

6. EXAMPLE: MATERIALS AND METHODS

6.1. CONDITIONS FOR RESTRICTION ENZYME DIGESTIONS

Restriction endonucleases were purchased from BRL (Bethesda Research Laboratories, Bethesda, Md.), IBI (International Biotechnologies, Inc., New Haven, Conn.), New England Biolabs (Beverly, Mass.), or U.S. Biochemical Corporation (Cleveland, Ohio).

Restriction enzyme digestions were carried out by suspending DNA in the appropriate restriction buffer, adding restriction endonuclease, and incubating for an appropriate period of time to ensure complete digestion. One unit of enzyme is defined as the amount required to completely digest 1.0 ug of phage lambda DNA in 1 hour in a total reaction mixture of 10 ul volume. Buffers used with the various enzymes are listed below:

Low salt buffer used for ClaI, HpaI, HpaII, and KpnI digestions consisted of: 10 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, and 10 mM dithiothreitol (DTT).

Medium salt buffer used for AluI, AvaI, SspI, TaqI, XmaI, and XmnI digestions consisted of: 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, and 10 mM DTT.

High salt buffer used for BamHI, EcoRI, EcoRV, NcoI, and SalI digestions consisted of: 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 150 mM NaCl, and 10 mM DTT.

The buffer used for SmaI digestions consisted of: 10 mM Tri-Cl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, and 10 mM DTT.

All restriction digestions were carried out at 37° C. except TaqI, which was carried out at 60° C.

6.2. GEL PURIFICATION OF DNA FRAGMENTS

After restriction enzyme digestions, DNA fragments of

sizes were separated and purified by gel electrophoresis in agarose using 0.1M Tris-Borate buffer (pH 8.0) containing 2 mM EDTA at 10 volts/cm. Agarose concentrations varied from 0.8% to 1.5% depending on the size of the fragments to be recovered. DNA bands were visualized by ethidium bromide fluorescence. DNA was recovered by electroelution of the chosen DNA fragment onto NA45 paper (Schleicher and Schuell, Keene, N.H.), followed by incubation of the NA45 paper at 68°

C. in a buffer consisting of 10 mM Tris (pH 8.0), 1 mM EDTA and 1M NaCl.

6.3 SYNTHESIS AND PURIFICATION OF OLIGONUCLEOTIDES

Oligonucleotides were synthesized on the 0.2 micromole scale, on an Applied Biosystems Inc. model 380B DNA synthesizer, using beta-cyanoethyl-phosphoramidite chemistry (Sinha, N. D., et al., 1984, Nucl. Acids Res. 12:4539-4544).

Oligonucleotides were purified by electrophoresis in a 0.4 mm thick 8% polyacrylamide gel in TBE buffer (0.01M Tris-borate, pH 8.2, 1 mM EDTA), run at approximately 1600 volts with a constant power of 75 watts. Oligonucleotide bands were visualized by negative shadowing over a PEI (polyethylene-imine) thin-layer chromatography plate under ultraviolet light, and the band of full length product was excised from the gel. The synthetic oligonucleotide was eluted in 0.3M sodium acetate pH 5.5, and was precipitated by the addition of two volumes of 100% ethanol, chilled to −20° C., and centrifuged at 14,000×g. The DNA pellets were dried under vacuum and dissolved in TE buffer (10 mM TrisCl, pH 7.4, 1 mM EDTA).

Phosphate groups were incorporated at the 5, terminus of the synthetic oligonucleotides using T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.). One microgram amounts of purified oligonucleotide were dissolved in 25 microliters of kinase buffer consisting of 70 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, with 1 mM adenosine triphosphate (ATP). This solution was incubated with 20 units T4 polynucleotide kinase for 30 minutes at 37° C.

Annealing of complementary strands was achieved by mixing the kinased strands and heating to 60° C. for 1 hour and cooling to room temperature. Annealed strands were used directly in ligation procedures.

6.4. CREATION OF FLUSH ENDS IN DNA FRAGMENTS

To create blunt ends for ligation, DNA termini with 5, overhangs resulting from digestion with restriction enzymes, were either filled-out by the action of the large fragment of DNA polymerase I (Klenow fragment), or removed by the action of mung bean nuclease. For filling-out with Klenow enzyme, approximately 1 microgram of DNA was treated with 1 unit of enzyme in 25 microliters of a buffer consisting of 50 mM Tris-Cl (pH 7.0), 10 mM $MgSO_4$, 0.1 mM dithiothreitol, and 50 ug bovine serum albumin per ml. A combination of deoxynucleotide triphosphates (dGTP, dCTP, dATP, dTTP) at a final concentration of 100 micromolar was also included. For digestion with mung bean nuclease, approximately 1 microgram of DNA was incubated with 1 unit of mung bean nuclease in 20 microliters in a buffer consisting of 50 mM sodium acetate (pH 5.0), 30 mM NaCl, and 1 mM $ZnSO_4$. Incubation was at 30° C. for 0.5 to 1 hour. mung bean nuclease and Klenow enzyme were obtained from New England Biolabs, Beverly, Mass.).

6.5. DNA LIGATION

All ligations were accomplished using T4 DNA ligase. T4 DNA ligase was purchased from BRL (Bethesda, Md.), U.S. Biochemical Corporation (Cleveland, Ohio), or Boehringer (Indianapolis, Ind.). One unit (U) of T4 DNA ligase is defined as the amount required to yield 50% ligation of HindIII fragments of bacteriophage lambda DNA in 30 minutes at 60° C. in 20 ul volume ligase buffer at a 5'-DNA termini concentration of 0.12 uM (300 ug/ml). DNA ligations were performed in ligase buffer consisting of 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP. Normally, DNA concentration ranged from 20-30 ug/ml. T4 DNA ligase was added at a ratio of 1 U per 20 ul reaction volume. Incubations were carried out for 18-24 hours. Temperatures used were 15° C. for cohesive end ligations, and 22° C. for blunt end ligations. If sufficient material was available, ligations were checked by analyzing a portion of the reaction mixture by agarose gel electrophoresis.

6.6. TRANSFORMATION OF PLASMID DNA

Plasmid constructions resulting from the ligation of fragments of circumsporozoite genes (or synthetic oligonucleotides related to such gene sequences) to cloning or expression vectors, were inserted into common laboratory strains of *Escherichia coli* by transformation techniques (for details, see Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Plasmid constructions were isolated and characterized first in *E. coli*, before transferring to Salmonella spp., because of the high transformation frequencies of *E. coli* K-12 relative to those of *S. typhimurium*. Plasmids were transferred into *S. typhimurium* LT-2 LB5010, a strain lacking several of the restriction systems known to exist in various Salmonella species and also containing a mutation in galE resulting in higher transformation frequencies (for a description of restriction systems of *Salmonella typhimurium*, see Bullas et al., 1980, J. Bacteriol. 141:275). Plasmids transformed into LB5110 were characterized for expression of the desired CS protein construct and, after verification by restriction enzyme analysis, plasmids were inserted into attenuated Salmonella by transduction techniques. LB5010 containing desired plasmid was grown in Luria broth (LB) to a density of 3×10^8 cells/ml, at which point D-galactose (to a final concentration of 1%) was added to the growth medium to induce synthesis of lipopolysaccharide (LPS). Following 1.5 hours of growth in the presence of D-galactose, bacteriophage P22 HT 105/1 int was added to the culture to a multiplicity of infection of one. Following adsorption of the phage, cells were immobilized in LB containing 0.7% agar. Phage were harvested and used to transduce plasmids into any attenuated Salmonella containing LPS as a component of the receptor for the transducing phage P22.

6.7. SYNTHESIS AND PURIFICATION OF CS IMMUNODOMINANT PEPTIDES

To demonstrate antigenicity or immunogenicity of subunit vaccines when compared to live attenuated Salmonella carrying CS proteins, two synthetic peptides, one representing two repeat units of circumsporozoite (CS) protein from *Plasmodium berghi* (DPAPPNAN; D-16-N) and the second representing 3 repeat units of CS from *Plasmodium falciparum* [(NANP)3] were synthesized by the solid phase method on an automatic peptide synthesizer (Applied Biosystem Model 430A). Synthetic peptides also served as capture antigens in ELISA assays.

The peptide chain was assembled on an insoluble polystyrene resin to which the carboxy terminal amino acid was attached through a special organic spacer known as "PAM" linker. Amino acids, protected with

t-butyloxycarbonyl (BOC), were coupled to the free alpha-amino groups on the growing peptide chain in an aprotic, polar solvent. Premature chain termination and peptide self-aggregation on the resin support was minimized by keeping the peptide chain dissolved in dimethylformamide during the coupling steps. The coupling reaction was monitored by a quantitative ninhydrin procedure which measures residual free alpha-amino groups on the peptide resin. All reactive side chains of individual t-BOC amino acids were protected with benzyl based protecting groups.

The completed peptides were cleaved from the resin, and all side-chain protecting groups were removed by anhydrous hydrofluoric acid (HF) treatment. After strong acid cleavage and deprotection, the reaction mixture was washed well with anhydrous ether and dissolved in dilute acetic acid. The resin was filtered, and the aqueous solution was collected and lyophilized to yield the crude peptide preparation.

The homogeneity of the peptide obtained from HF cleavage was evaluated on a micropore HPLC (high performance liquid chromatography) system (Applied Biosystems Model 130A separation system) using a reverse phase column. If HPLC analysis revealed a single major peak, no further purification was performed. If the HPLC chromatogram showed a mixture of peaks, preparative HPLC was performed to separate peaks. The purified product was subjected to acid hydrolysis for amino acid analysis to verify the amino acid composition. The synthetic peptides were sequenced by automated Edman degradation with a protein sequenator (Applied Biosystems Model 477A) equipped with a fully automatic on-line phenylthiohydantoin (PTH) analyzer (Model 120A).

6.8. PEPTIDE-KEYHOLE LIMPET HEMOCYANIN CONJUGATION PROCEDURE

Synthetic peptides were coupled to keyhole limpet hemocyanin (KLH) by glutaraldehyde cross-linking. KLH (obtained from Calbiochem, San Diego, Calif.) was dialysed against 0.1M sodium bicarbonate buffer (pH 9.6) and adjusted to 1 mg/ml in the same buffer. Peptides were made in water or 0.1M sodium bicarbonate buffer at 4 mg/ml. Equal volumes of protein and peptide solutions were mixed and rotated for 1 hour at room temperature. Four microliters of 25% aqueous solution of glutaraldehyde was added and rotated for another 24 hours, followed by another 25 microliters of 25% glutaraldehyde and rotation for 72 hours at room temperature. The conjugated material was dialysed against phosphate-buffered saline for 24 hours. Peptide-KLH conjugates were tested in ELISA, as previously described (Egan et al., 1984, Science 236:453). Initially, plates were coated with different concentrations of conjugates, and the concentration that reacted well with the appropriate monoclonal antibodies was selected for coating plates in all future experiments.

6.9. POLYACRYLAMIDE GEL ELECTROPHORESIS

To analyze proteins by polyacrylamide gel electrophoresis (PAGE), cells from 1 ml of culture were washed and resuspended in 100 microliters of a lysing buffer (0.2M Tris-HCl buffer containing 5% SDS, 0.025% bromophenol blue, 0.1 to 1M 2-mercaptoethanol, and 20% glycerol), and heated for 5 minutes at 100° C. Most analyses were performed using the Bio-Rad Mini Protean Gel system (Redmond, Calif.). Gels were 1.5 mm thick, and the separating gel contained 15% acrylamide, with an acrylamide to bis-acrylamide ratio of 30:0.8, 0.375 M Tris-HCl (pH 8.8), and 0.1% sodium dodecyl sulfate (SDS). The stacking gel contained 4.8% acrylamide with the same 30:0.8 ratio of acrylamide to bis-acrylamide, 125 mM Tris-HCl (pH 7.0), and 0.1% SDS.

Ten to fifteen microliters of samples (containing 5–20 micrograms protein) were applied to each lane. Following electrophoresis, gels were stained for at least 1 hour with 0.125% Coomassie blue in ethanol:acetic acid:water (5:1:5), then destained in the same solvent system without the dye. Pre-stained low molecular weight standards (ovalbumin, 43,000; alpha-chymotrypsinogen, 25,700; B-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6,200; insulin, 2,300 and 3,400; obtained from Bethesda Research Laboratories, Gaithersburg, Md.) were also subjected to electrophoresis to assist in the determination of the relative molecular weight of the observed proteins. An unstained duplicate gel without staining was used for western analysis.

6.10. WESTERN BLOT AND ISOELECTRIC FOCUSSING ANALYSIS

Samples separated by PAGE were transferred electrophoretically onto nitrocellulose membranes in a Hoeffer Transphor apparatus at 0.45 milliamps for 90 minutes in 25 mM Tris, 384 mM glycine (pH 8.8) at room temperature. Once protein transfer was complete, nitrocellulose membranes were soaked in BLOTTO (5% non-fat dry milk in phosphate-buffered saline) at 37° C. for 1 hour. Membranes were probed with a pre-determined concentration of monoclonal antibodies against *P. berghei* or *P. falciparum* CS protein repeat regions for 1 hour at 37° C. and washed with BLOTTO for 20 minutes at 37° C. Bound antibodies were detected by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard and Perry, MD) at 1:250 dilution in BLOTTO for 1 hour at 37° C. Blots were washed three times with PBS, and developed with PBS containing 0.01% hydrogen peroxide, 0.06% 4-chloro-1 napthol (Sigma Chemical Co., Mo.), in methanol for 20 minutes at room temperature. The reaction was stopped by transferring the filters to distilled water. The filters were dried by blotting.

For isoelectric focussing of proteins in polyacrylamide gels, approximately 10–50 micrograms of total cell proteins were applied to the gel. Cell extracts were prepared by harvesting bacteria in the logarithmic phase of growth, and concentrating them fifty-fold by centrifugation and resuspension in 0.5 ml of 10 mM TrisHCl, pH 8.0, containing 1 mM EDTA and 20 ug lysozyme per ml. After digestion with lysozyme, extracts were subjected to sonication. Whole cells and cellular debris were removed by centrifugation at 12,000×g, and the supernatant samples, in a volume of 10–50 microliters, were applied to an isoelectric focussing gel in a sample buffer consisting of 10% v/v glycerol, 2% carrier ampholytes (pH 3–10) and 0.001% methyl red. Focussing gels consisted of 7.5% acrylamide containing 2% ampholytes C (pH 3–10; purchased from BioRad, Richmond, Calif.). Focussing of proteins was carried out over a 4–5 hour period at 2000 V. Focussed proteins were transferred to nitrocellulose by electroblotting, followed by visualisation with monoclonal antibody to the CS protein repeat region as described supra for the western blot technique.

6.11. COLONY BLOT SCREENS FOR CIRCUMSPOROZOITE PROTEIN EXPRESSION IN *E. COLI*

E. coli transformant colonies were screened for expression of the immunodominant CS epitope by lysing cells retained on nitrocellulose filters by exposure to chloro form vapors for a period of 20 minutes. Lysed colonies were washed off the nitrocellulose filter by immersing the filters in a blocking solution consisting of 50 mM Tris-Cl. pH 8.0, containing 0.15M NaCl. 5 grams of Carnation instant dried milk (BLOTTO) per 100 ml of solution. 1 microgram boiled RNase per ml. 1 microgram DNase per ml. and 200 micrograms egg white lysozyme per ml. Filters were washed further in BLOTTO. and monoclonal antibody 3.28 (Egan et al., 1987, Science 236:453), capable of recognizing the immunodominant repeated epitope of *P. berghei*, or a mixture of two or more monoclonal antibodies, mAb 4D9.1P or mAb 565, reacting with the repeat epitope of *P. falciparum* (Dame et al., 1984. Science 225:593), was added to a concentration of 10–100 nanograms per ml of blocking solution. Filters were incubated for 1 hour at 37° C., followed by washing in BLOTTO. The bound antibody was amplified by addition of rabbit anti-mouse IgG antibody, and incubation for another hour at 37° C. The signal was developed by incubation with goat anti-rabbit IgG coupled to horseradish peroxidase. Horseradish peroxidase was visualized by reaction in the presence of 0.06% 4-chloro01-napthol (w/v) and 0.15% hydrogen peroxide (v/v).

6.12. ENZYME-LINKED IMMUNOABSORBENT ASSAY FOR SERUM ANTI-CS AND ANTI-LT-B ANTIBODIES

To measure serum antibodies, 96 well polystyrene plates (NUNC) were coated with 1 ug/ml of LT-B or 5 ug/ml of D-16-N-KLH, a synthetic peptide representing two repeat units of *Plasmodium berghei* CS protein coupled to KLH by glutaraldehyde cross-linking. Each well received 0.1 ml of antigen in 0.1M carbonate/bicarbonate buffer (pH 9.6). Plates were incubated at 37° C. in a humidified incubator for 18 hours, before being washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 0.1% gelatin in PBS for 60 minutes at room temperature. Plates were washed 3 times with PBS-T, and serial dilutions of sera were added and incubated for 90 minutes at room temperature. Goat anti-LT-B or mAb 3.28, or pools of mAb 4D9.1P and mAb 565, were used as positive controls in assays. Plates were washed as before, and pre-optimized concentrations of alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin (at a 1:3000 serum dilution; Tago, Burlingame, Calif.), and goat anti-mouse immunoglobulin (at a 1:5000 serum dilution; Tago, Burlingame, CA) were added to appropriate wells and incubated for 60 minutes at room temperature. Plates were washed again, and 100 microliters of substrate solution (p-nitrophenyl phosphate at 1 mg/ml in diethanolamine buffer, pH 9.6) was added to each well. The signals were developed for 60 minutes at room temperature, and read in a Bio-Tek automatic ELISA reader using dual wavelengths at 410 nm and 690 nm, blanking on air.

6.13. CHALLENGE OF MICE WITH LIVE SPOROZOITES

Sporozoites of *Plasmodium berghei* were obtained from salivary glands of infected female *Anopheles* mosquitoes. The salivary glands were dissected out and collected in ice-cold tissue culture medium M199 supplemented with 10% normal mouse serum. The glands were gently triturated in a loose-fitting glass grinder. Sporozoites were separated from mosquito tissue debris by centrifuging triturated glands at 500 rpm in a Sorvall SS34 rotor for 3 minutes at 4°C and collecting parasite-containing supernatant. For maximum yield, the extraction was repeated twice. The concentration of sporozoites was determined by counting the parasites in a hemocytometer.

Seven to 14 days after the final vaccination, animals were divided into sub-groups and challenged with a low dose (500) or high dose (2000) of sporozoites of *Plasmodium berghei*. Starting from day 3 after challenge, mice were examined daily for mortality and for detectable parasitemia in Giemsa-stained blood smears. All patent infections usually appeared within 10 days after challenge.

7 EXAMPLE: EXPRESSION VECTORS FOR PLASMODIUM CIRCUMSPOROZOITE PROTEIN GENES

7.1. DNA SEQUENCE OF THE GENE FOR THE pi P. BERGHEI CIRCUMSPOROZOITE PROTEIN

The gene encoding approximately 75% of the carboxy-terminal portion of the circumsporozoite protein of *P. berghei* was obtained as an 1140 base pair EcoR1 restriction fragment in the plasmid vector pUC8 (Veira, J. and Messing, J., 1982, Gene 19:259). The DNA sequence of the fragment has been previously determined (Weber et al., 1987, Experimental Parasitol. 63:295), and is depicted in FIG. 1, with the deduced amino acid sequence listed in FIG. 2. In addition to the sequence shown in FIG. 1, an EcoRI-TaqI oligonucleotide linker was used during the initial cloning and is contained within the EcoR1 restriction fragment. This adapter, or linker region, contains nucleotide sequences which comprise recognition sites for restriction endonuclease cleavage. These restriction enzyme sites can be used as convenient cleavage sites for subcloning the gene sequence into expression vectors. The sequence of the adapter region is as shown (the adapter sequences are underlined):

circumsporozoite protein gene

TaqI NruI XmnI

5' AATTCGAACCCCTTCG CGA . . . TAT CGCGAAGGGGTTCGAATT 3'

XmnI NruI TaqI

7.2. CONSTRUCTION OF PLASMID pPX100. EXPRESSING LT-B UNDER THE CONTROL OF THE lac OPERON

Plasmid pJC217 encodes 100 amino acids of mature *E. coli* enterotoxin subunit B, LT-B, and also encodes a 20 amino acid amino-terminal signal sequence which is cleaved from the mature molecule. Plasmid pJC217 was obtained by insertion of approximately 800 base pairs of LT-B DNA (the sequence of which is shown in FIG. 3), from a genomic fragment of enterotoxigenic *E. coli* H10407, into the HindIII site of plasmid vector pUC8.

To obtain an expression vector in which fusions of the circumsporozoite protein of *P. berghei* with various regions of LT-B can be created, plasmid pJC217 was modified to delete extraneous restriction sites associated with the polylinker region of pUC8. By digesting pJC217 with the restriction enzyme EcoR1 and religating the reaction products, a deletion of 180 base pairs of DNA including the DNA specifying the restriction sites of the polylinker region was obtained, resulting in plasmid vector pPX100 (FIG. 4). In this configuration, LT-B is controlled by the lac operon promoter of *E. coli*. After transfer of the plasmid into a suitable bacterial host, e.g., *E. coli* JM103 (Clements et al., 1984, Infect. Immun. 46:564). LT-B transcription can be induced by inclusion of isopropyl-thio-beta-D-galactoside (IPTG) in the growth medium of the recombinant bacteria. The DNA sequence of LT-B is depicted in FIG. 3; restriction enzyme sites useful in creating fusion protein molecules are indicated.

7.3. EXPRESSION OF THE CIRCUMSPOROZOITE PROTEIN OF *P. BERGHEI* IN *E. COLI*, AS A FUSION PROTEIN WITH BETA-GALACTOSIDASE

To obtain expression of the circumsporozoite protein gene in *E. coli*, plasmid pUC8 containing the 1.1 kilobase pair EcoRI CS fragment was digested with either NruI or XmnI under standard conditions for those enzymes. A 670 base pair XmnI fragment was purified by electrophoresis through a 1% agarose gel and eluted from agarose by electrophoresis onto NA45 paper (Schleicher and Schuell, Keene, N.H.). The DNA fragment was eluted off of the NA45 paper by incubating the paper in a 250 microliter volume of 10 mM Tris hydrochloride buffer, pH 8.0, containing 1M NaCl and 5 mM EDTA. DNA was precipitated from the NaCl buffer by addition of two volumes of ethanol, and the DNA pellet was obtained by centrifugation at 12,000×g for 5 minutes at 4° C. The 1.1 kilobase pair NruI fragment was purified in the same fashion. The XmnI and NruI DNA fragments were each redissolved in a sufficient volume of 10 mM Tris hydrochloride containing 10 mM EDTA to yield a final concentration of approximately 1 microgram per microliter. Plasmid vector pUC8 was digested with HincII and mixed either the with purified XmnI or the NruI fragment, and the DNA ends were joined with T4 DNA ligase. The ligation products were transformed into *E. coli* JM103, with selection for resistance to ampicillin.

7.3.1. DETECTION OF TRANSFORMANT COLONIES EXPRESSING THE REPEATED EPITOPE OF THE CIRCUMSPOROZOITE PROTEIN

Transformant bacterial colonies reacting with mAb 3.28, an antibody which recognizes the immunodominant repeated epitope of *P. berghei* CS, were detected by the colony blot method described in Section 6.11, following induction of lac operon expression by transfer of the colonies to an LB agar plate containing 1 mM IPTG.

After transformation with the plasmid resulting from religation of pUC8 restricted by the enzyme HincII in the presence of either the 1.1 kilobase pair NruI CS fragment or the 670 base pair XmnI CS fragment, as described above, approximately 10% of the resulting transformants demonstrated significant color development when reacted with mAb 3.28. Among the reactive colonies from each ligation, several candidates were retained and analyzed further. Plasmids isolated from each candidate colony were subjected to restriction enzyme analysis to confirm the presence of characteristic cleavage sites. Plasmid pPX1512 results from the linkage of the 670 base pair XmnI fragment in frame with the first eleven amino acids encoded by the polylinker region of pUC8, and reads in-frame through the 223 amino acids encoded by the DNA of the XmnI fragment through the following sixty-eight amino acids of the beta-galactosidase peptide, termed the lac alpha peptide, encoded in the pUC8 vector. Plasmid pPX1514 results from the linkage of the 1.1 kilobase pair NruI fragment into the HincII site of pUC8, and contains a fusion of the first eleven amino acids of the polylinker region to 272 amino acids of the circumsporozoite protein. The translated protein of pPX1514 terminates with the translation termination codon TAA supplied in the circumsporozoite protein sequence.

7.4. FUSION OF THE *P. BERGHEI* CIRCUMSPOROZOITE PROTEIN TO THE CARRIER POLYPEPTIDE LT-B

The circumsporozoite gene of *P. berghei* was linked to the gene for the B subunit of *E. coli* enterotoxin, LT-B, at three separate loci within the gene sequence. As diagrammed in FIGS. 5 and 6, pPX100 was cut with three separate restriction enzymes by standard techniques, and the resulting cohesive ends were modified so that blunt-end ligation of either the XmnI or the NruI fragment containing the repeat regions of the circumsporozoite gene would result in in-frame fusion of the CS gene with regions of the LT-B gene contained in pPX100. First, pPX100 was cut with ClaI and the cohesive ends "filled- out" by the action of the Klenow fragment of DNA polymerase I in the presence of dCTP and dGTP. When the 670 base pair XmnI CS fragment was ligated to the modified ClaI site, an LT-B fusion protein resulted. This protein, as encoded in pPX1515, comprises the first 30 amino acids of mature LT-B fused to 223 amino acids of the CS gene, followed by an out-of-frame readthrough of twenty-eight amino acids derived from LT-B DNA downstream from the ClaI site. When the 1.1 kilobase pair NruI fragment was ligated to the modified ClaI site, a protein resulted in which the first thirty amino acids of mature LT-B were fused with 272 amino acids of the CS gene, with translation terminating with the termination codon of the CS gene.

Equivalently, pPX100 was digested with XmaI and treated further with mung bean nuclease to remove the overhanging 5' terminus. The 1.1 kilobase pair NruI CS fragment was ligated to this modified site, yielding plasmid pPX1523. The resulting fusion protein contained sixty amino acids of mature LT-B linked to 223 amino acids of the CS gene, followed by an out-of-frame read-

through of four amino acids derived from LT-B DNA. To fuse the CS protein sequences to the carboxy-terminal amino acid of mature LT-B. pPX100 was digested with the restriction enzyme SpeI. The resulting cohesive termini were partially filled in by the action of the Klenow enzyme of DNA polymerase I in the presence of dCTP. and the remaining overhang was digested with mung bean nuclease. Blunt-end ligation of either the 1.1 kilobase pair NruI or the 670 base pair XmnI CS fragment resulted in the fusion proteins depicted in FIG. 6. Plasmid pPX1525 encodes a LT-B/CS fusion protein in which 100 amino acids of mature LT-B is linked to 223 amino acids of the CS protein, followed by forty amino acids read out-of-frame, which are derived from DNA present in the LT-B clone (outside the coding region of LT-B).

7.5. MANIPULATION OF TRANSCRIPTIONAL PROMOTER AND TRANSLATION INITIATION SIGNALS TO OBTAIN HIGHER LEVELS OF LT-B/CS FUSION PROTEIN EXPRESSION

Increased expression of LT-B CS fusion proteins was obtained by inserting the fused gene sequences directly downstream from either of two strong promoter sequences known to function well in *E. coli*. The tac promoter results from the fusion of the −35 region of the trp operon promoter with the −10 region of the UV5 lac promoter (DeBoer, H., et al., 1982. in Promoter Structure and Function, Rodriguez, R. L. and Chamberlain, M. J., eds., Praeger Publishing, New York). The tac promoter is controlled by the lacI gene product, a repressor which binds to the lac region of the DNA and inhibits transcription by RNA polymerase. In the presence of the lacI gene product, gene expression can be induced by IPTG present in the bacterial growth medium. The P_L promoter of coliphage lambda controls leftward transcription of mRNA during lytic growth of the virus in its natural host. *E. coli* (Shimatake, H. and Rosenberg, M., 1981, Nature 292:128). The PL promoter is repressed during lysogeny by the cI gene repressor protein. When either the tac promoter or the PL promoter is used to drive gene transcription in a closely related enteric host bacterium such as Salmonella spp., gene transcription is constitutive unless appropriate repressor functions are also introduced. Gene transcription results in constitutive synthesis of proteins encoded by DNA downstream from the promoter sequences. Synthesis of antigenic proteins in a Salmonella spp. host, such as the LT-B/CS fusion proteins described in Section 7.4, results in constant presentation of the antigenic molecules during the period of time the attenuated bacteria can replicate within the animal host, either in the experimental test animal such as the mouse, or in humans. A high level of specific protein synthesis relative to endogenous bacterial host proteins favors host immune response to the recombinant DNA-derived proteins.

To demonstrate increased synthesis of equivalent proteins expressed in Salmonella spp., each of the family of LT-B/CS fusion proteins diagrammed in FIG. 6 was inserted directly downstream from either the tac promoter (FIG. 7) or the P_L promoter (FIG. 8). The translation initiation signals derived from LT-B are retained within the EcoRI-HindIII fragments encoding the LT-B/CS fusion proteins and include a ribosome binding site (S/D sequence) and the translation start codon of LT-B at the 5' terminus of the gene sequence (FIG. 6). Plasmid vector pKK223 (purchased from Pharmacia Molecular Biologicals, Piscataway, N.J.) contains the tac promoter, but lacks suitable translation start sequences downstream from the promoter. Plasmid pKK223 was digested with restriction enzymes EcoRI and HindIII. The plasmid vector fragment was purified by electrophoresis through agarose and was ligated to purified EcoRI-HindIII restriction fragments derived from either pPX1515, pPX1523, or pPX1525. Transformants obtained in *E. coli* JM103 were screened for production of proteins reactive to the specific anti-CS monoclonal antibody, mAb 3.28, after transfer of bacterial colonies to LB agar containing 1 mM IPTG. Reactive colonies were purified, and DNA isolated from them was screened further by restriction enzyme analysis. Those colonies showing the expected DNA restriction patterns were retained. As diagrammed in FIG. 7, plasmid pPX1528 was obtained in this fashion.

To obtain expression of an equivalent family of fusion proteins controlled by the P_L promoter of coliphage lambda, the EcoRI-HindIII restriction fragments derived from pPX1515, pPX1523, and pPX1525 were treated with the Klenow fragment of DNA polymerase I in the presence of dATP, dTTP, dCTP, and dGTP, to create blunt ends at both the EcoRI end and the HindIII end of the fragments (FIG. 8). By ligating the blunt-ended fragments into the HpaI site of plasmid vector pP_L-lambda (Pharmacia), a series of plasmids encoding fusion proteins controlled by the P_L promoter was obtained by transforming *E. coli* N99 cI+. Plasmid pPX1601 was derived from a fragment of pPX1515, ligated into expression vector pP_L-lambda (FIG. 8). Transformants were screened in *E. coli* for the presence of unique restriction fragments. Suitable candidates were then transformed into *E. coli* strain N4830, which is lysogenic for a defective bacteriophage lambda harboring a temperature-sensitive repressor allele, cI857. In this *E. coli* host strain, genes controlled by the lambda P_L promoter are inducible by switching the temperature of the growth medium from 32° C. to 42° C. As depicted in FIG. 8, in this configuration, translation initiation signals are supplied by the S/D sequence and translation initiation codon (ATG) derived from LT-B. The vector also encodes part of the bacteriophage lambda N gene, whose transcription is controlled by the P_L promoter. By inserting the blunt ended EcoRI-HindIII fragment of the LT-B/CS fusion proteins in the HpaI site, the N gene terminates with the TGA codon immediately after the S/D sequence as shown in FIG. 8.

7.6. CONSTRUCTION OF EXPRESSION VECTORS TO PROMOTE TRANSCRIPTION FROM THE LAMBDA P_L PROMOTER OF GENES ENCODING CIRCUMSPOROZOITE PROTEINS

To provide suitable translation initiation signals downstream from the P_L promoter, an oligonucleotide was designed to be inserted into the HpaI site of pP_L lambda. This oligonucleotide consists of 53 base pairs; the sense strand is diagrammed in FIG. 9. The synthetic oligonucleotide is designed so that translation of the N gene terminates at the TAA immediately upstream from the S/D sequence. In addition, the presence of KpnI and NcoI sites bracketing the S/D sequence allows for the facile insertion of short synthetic oligonucleotides in order to randomize the base sequence around the S/D sequence and obtain increased translation efficiency. In addition, the oligonucleotide specifies three restriction

sites, namely, NcoI, StuI, and EcoRV, which allow for insertion of heterologous sequences in all three reading frames. The NcoI site encompasses the required ATG initiation codon, which can be supplied as a blunt end by filling out the cohesive NcoI end with Klenow enzyme in the presence of all four deoxynucleotides. Moreover, the sequence encodes translation stop codons in all three reading frames, so that predictable termini for fusion proteins are obtained.

Plasmid pPX1529 was obtained by ligating vector pPX1600 (after treatment with NcoI and filling out the cohesive ends with Klenow enzyme) to the 670 base pair XmnI fragment encoding the *P. berghei* CS protein repeat epitope and Regions I and II (FIG. 10). In addition, by treating the 670 base pair XmnI fragment with NruI, followed by purification and ligation of the resulting DNA fragment into the filled NcoI site of pPX1600, a plasmid (pPX1531) was obtained in which the codon for arginine (CGA), derived from the proper *P. berghei* CS DNA sequence, was fused directly to an amino terminal methionine. Thus, no extraneous amino acids resulting from the translation of linker DNA, other than the initial methionine, was obtained. A seven amino acid carboxy terminal addition was obtained in this fusion, resulting from the translation of codons derived from the synthetic insert of pPX1600.

7.7. INSERTION OF EPITOPES OF *P. FALCIPARUM* AND *P. BERGHEI* INTO THE pPX1600 EXPRESSION VECTOR

To express the CS repeat regions of either the rodent malaria parasite *P. berghei* or the human malaria parasite *P. falciparum* at appropriately high levels in *E. coli* and Salmonella spp., synthetic complementary oligonucleotide strands were designed as diagrammed in FIG. 11. The oligonucleotide encoding the *P. falciparum* repeat region was based on the consensus sequence ASN ALA ASN PRO (NANP) repeated four times. In addition, the oligonucleotide was designed so that asymmetrical HinfI cohesive termini were formed at each end. By treating the annealed complementary strands with T4 polynucleotide kinase and T4 DNA ligase, head to tail polymerization of the repeated epitope was achieved. Creating blunt ends from the resulting single HinfI cohesive ends and ligating the fragment into the blunt-ended NcoI site of pPX1600 resulted in a series of transformants of *E. coli* strain N99 cI$^-$ containing the *P. falciparum* CS repeat epitope. (This strain of *E. coli* expresses the cI$^+$ temperature-insensitive wild-type lambda repressor, and is lysogenic for bacteriophage lambda.) By screening the transformants with appropriate restriction enzymes, clones having from one (monomer) to four (tetramer) copies of the *P. falciparum* oligonucleotides in the correct orientation with respect to the P_L promoter were obtained. These plasmids were transformed into the *E. coli* expression host N4830 (cI857), where expression of the epitope induced by temperature shift was confirmed by colony blot analysis and western blot analysis.

Concurrently, an oligonucleotide encoding two repeats of the *P. berghei* consensus octapeptide epitope (ASP PRO ALA PRO PRO ASN ALA ASN: DPAPPNAN) was synthesized. As described supra, two complementary strands were designed so that head to tail polymerization could occur upon ligation of annealed strands following treatment with T4 polynucleotide kinase and T4 DNA ligase. Ligated oligonucleotides were treated with Klenow enzyme in the presence of deoxynucleotides and the resulting blunt-ended family of fragments were ligated to the filled-out NcoI site of pPX1600. Transformant colonies were selected in *E. coli* N99 (cI+) and analyzed by restriction enzyme digestion. Monomeric to tetrameric inserts were isolated and characterized further for expression of the immunodominant epitope by screening plasmids for induction following transformation into the *E. coli* expression host N4830.

To express the *P. falciparum* CS protein gene from a full length clone lacking only the sequence encoding the putative 16 amino acid signal sequence, a StuI-RsaI DNA fragment was cloned directly into the StuI site of plasmid pPX1600. The sequence data of Dame et al. (1984, Science 225:593) was used to establish the CS protein reading frame within the restriction fragment and to predict the expression of the gene inserted into the StuI site of pPX1600. In this configuration, the full length mature gene is expressed from the P_L promoter of the vector, and the initiating ATG (methionine) is that of the vector. The resulting plasmid was named pPX1534 and is diagrammed in FIG. 12.

7.8. CONSTRUCTION OF VECTORS WHICH EXPRESS LT-B/CS FUSION PROTEINS, BY INSERTION OF SYNTHETIC OLIGONUCLEOTIDE REPEAT SEQUENCES

To construct plasmid vectors which encode fusions of the *P. falciparum* or *P. berghei* immunodominant CS epitope to portions of the LT-B sequence, synthetic oligonucleotides encoding the epitope were ligated (following filling out of the recessed 3' ends) into the ClaI site of pPX100 (FIG. 4). The ClaI site was filled out by the action of Klenow enzyme in order to maintain the reading frame of the CS protein. In particular, the oligonucleotide designed to encompass the *P. falciparum* repeated epitope is shown below with the translated amino acid sequence below it:

```
5'...GAT CCG AAC GCT AAC CCG AAC GCT AAC CCG AAC GCT
3'...    GGC TTG CGA TTG GGC TTG CGA TTG GGC TTG CGA
    Asp  Pro [Asn  Ala  Asn  Pro ][Asn  Ala  Asn  Pro ][Asn  Ala

    AAC CCG AAC GTT     ...3'
    TTG GGC TTG CAA CTA...5'
    Asn  Pro ][Asn  Val
```

This sequence includes three repeats of the consensus tetrapeptide [Asn-Ala-Asn-Pro]. This double-stranded oligonucleotide with sticky ends was blunted by Klenow enzyme and ligated into the ClaI site of pPX100 to yield plasmid pPX1532, in which two 48-mer oligonucleotide units were cloned in-frame with the first 30 amino acids of LT-B, followed by out-of-frame reading of 28 amino acids derived from the LT-B gene sequence. The recombinant clones were isolated and the plasmid DNA characterized in *E. coli* strain JM103.

7.9. EXPRESSION OF CIRCUMSPOROZOITE PROTEIN EPITOPES IN *E. COLI* AND IN SALMONELLA SSP.

Plasmids were designed as described supra to express variants of the circumsporozoite epitopes of either *P. falciparum* or *P. berghei*. As described therein, the expression of these proteins or portions of these proteins, was designed to be controlled by several different promoter systems known to function and drive gene expression in *E. coli*. As such, plasmid constructions were first isolated and tested in commonly available laboratory strains of *E. coli* such as JM103, which is a suitable strain for studying control of gene expression controlled by the lac promoter and repressor, and such as N99 (cI$^-$) and N4830 (cI857), which are suitable *E. coli* hosts for examining gene expression controlled by the P_L promoter. Because overexpression of gene products can often lead to deleterious effects on bacterial cell growth, control of gene expression can be important in obtaining the desired expression plasmid construction.

After desired plasmids were obtained in suitable *E. coli* hosts, plasmids carrying variants of the CS genes were transferred into several different species of Salmonella. Expressing plasmids were transferred into either *S. enteriditis* serotype dublin (commonly known as *S. dublin*) SL1438 (ATCC Accession No. 39184), *S. typhimurium* SL3261, or into *S. typhi* Ty523 or Ty541. (*Salmonella typhimurium* strain SL1479, ATCC Accession No. 39183, and *S. typhi* strain Ty531, ATCC Accession No. 39926, are other strains readily available for use.) The attenuated mouse virulent strains SL1438 and SL3261 carry a chromosomal deletion of the aroA gene (Hoiseth, S. K. and Stocker, B. A. D., 1981, Nature 291:238). The attenuated *S. typhi* strain Ty523 carries a chromosomal deletion of the aroA gene, and Ty541 carries an additional deletion of the purA gene. To test whether the chosen promoters function in each of the three host Salmonella strains, plasmids pPX1515, pPX1528, and pPX1601 were transformed into *S. typhimurium* LT-2, strain LB5010 (a non-restricting, transformable mutant), from which P22 phage lysates (Schmeiger, 1972, Mol. Gen. Genetics 119:75) were obtained to transduce each of the plasmids into the desired strain of Salmonella. Expression of the LT-B/CS fusion plasmids was examined in each of the bacterial cultures by the western technique of Section 6.10. As shown in FIG. 15, expression of the fusion protein controlled by each promoter was obtained in each of the host Salmonella strains. FIG. 15 shows that expression of the LT-B/CS fusion protein containing the *P. berghei* CS protein sequence, controlled by either the lac promoter, the tac promoter, or the lambda P_L promoter, was observed in each of three attenuated Salmonella strains: SL3261, SL1438 and Ty523.

In addition, the expression of the *P. falciparum* CS protein repeat epitope fused to the first 30 amino acids of the mature LT-B protein was demonstrated (FIG. 16). Cell extracts of the Salmonella strains SL3261, SL1438, and Ty523, carrying pPX1532, were analyzed by isoelectric focussing as described in Section 6.10. The *P. falciparum* epitope was shown to be expressed in each of the strains, by binding anti-CS protein monoclonal antibodies as described in Section 6.10.

8. EXAMPLE: VACCINATION AGAINST MALARIA WITH ATTENUATED RECOMBINANT SALMONELLA WHICH EXPRESS CS PEPTIDES

As demonstrated in Section 7.9, the circumsporozoite proteins of either *P. falciparum* or *P. berghei* can be expressed at significant levels in several different Salmonella species including *S. dublin*, *S. typhi*, and *S. typhimurium*. The lac, tac, and P_L promoters each is capable of driving the expression of LT-B/CS fusion proteins or portions of CS proteins. The immunodominant CS peptides encoded by synthetic oligonucleotides can be efficiently expressed when regulated by the P_L promoter and translation initiation signals supplied by the expression vector. To test the vaccine potential of each of the plasmid constructs in an animal model system, each of the Salmonella bacteria carrying plasmids expressing CS peptides was used to vaccinate mice.

8.1. IMMUNOGENICITY IN MICE OF RECOMBINANT SALMONELLA WHICH EXPRESS CS PEPTIDES

The ability of recombinant Salmonella expressing CS peptides to elicit antibody against CS proteins was demonstrated by the detection of specific anti-CS antibodies in the sera of mice vaccinated with the recombinant bacteria. Six week old female C57B1/6 mice (Taconic labs) were used throughout this study. Log phase cultures of appropriate bacteria were washed three times in sterile phosphate-buffered saline (PBS), and resuspended to 10^8 cells per ml in PBS. Mice were divided into groups of 5–10 and injected intraperitoneally (i.p.) with 0.1 ml of the appropriate bacterial cell suspension (10^7 cells) Alternatively, mice were inoculated orally with doses of 10^{10} recombinant bacteria on day 0, followed by a second dose of 10^{10} bacteria on day 3. Control groups received either Salmonella strains expressing LT-B proteins from recombinant plasmids, or Salmonella strains carrying pUC8 or pUC18 parental plasmids. Each mouse was bled before immunization, and sera was stored at −70° C. for future analysis. Mice initially vaccinated by the i.p. route were bled again on week 4 and boosted with 10^8 bacteria i.p. Mice which had been vaccinated orally were boosted on week 4 with 10^{10} bacteria, followed three days later with a second boosting dose. Serum samples were tested for the presence of anti-CS antibody and anti-LT-B antibody, by use of an enzyme-linked immunoabsorbent assay (ELISA), as described in Section 6.12.

The actual OD (optical density) values of control and experimental sera at 1:160 dilution are presented in FIG. 17. Titers were determined based on a cutoff OD corresponding to the mean OD of pre-immune sera plus 3 standard deviations. A four fold rise in titer from pre- to post-immune sera was considered significant. *Salmonella dublin* SL1438 carrying pPX1528 or pPX1601 plasmids induced significant anti-CS primary antibody response but no anti-LT-B antibody response; both plasmids express an LT-B/CS fusion protein. In contrast, pPX1527 in SL1438 (expressing the LT-B gene) stimulated anti-LT-B antibodies. A slightly higher anti-CS response was observed with pPX1601 in SL1438 (expressing the CS gene under the control of the P_L promoter) than that with pPX1528 in SL1438 (expressing the CS gene under the control of the tac promoter), suggesting that a higher level of expression may be very critical to elicit greater antibody response in this system.

Boostable response was observed for each of the plasmid constructions, with the highest titer being observed with the bacteria expressing the largest amount of CS protein or LT-B/CS fusion protein. Mice that had been vaccinated with *Salmonella dublin* SL1438 expressing LT-B/CS fusion protein driven by the lac promoter showed no significant response after either primary or secondary vaccination, suggesting that the expression level of the fusion protein was too low.

8.2. PROTECTION AGAINST PLASMODIUM INFECTION IN MICE AFTER IMMUNIZATION WITH RECOMBINANT SALMONELLA EXPRESSING CS PEPTIDES

The efficacy of the recombinant Salmonella strains expressing CS proteins for use as vaccines was demonstrated in the mouse model system, by showing protection against Plasmodium infection in immunized mice upon sporozoite challenge. Although expression of the *P. falciparum* immunodominant epitope can be demonstrated in *S. typhi* and other Salmonella, mice cannot be infected with sporozoites of *P. falciparum*. To demonstrate the ability of recombinant *Salmonella dublin* SL1438 carrying LT-B/CS fusion protein-encoding plasmids or CS protein-encoding plasmids to elicit protection, mice previously vaccinated with recombinant Salmonella were challenged with either a low dose (1000/mouse) or a high dose (10^4/mouse) of *P. berghei* sporozoites, by tail vein injection. As a control, unvaccinated mice were also challenged.

S. dublin SL1438, carrying either plasmids pPX1528 (tac-promoted) or pPX1601 (P_L-promoted) expressing the LT-B/CS fusion protein, or carrying pPX1529 expressing the immunodominant repeat region of the CS protein, are capable of eliciting a protective response. In control experiments, vaccination at week zero and boosting at week four with either 10 micrograms D-16-N-KLH (DPAPPNAN-KLH: Egan et al., 1987, Science 236:453) or with 10 micrograms of partially purified LT-B/CS fusion protein derived from *E. coli* (strain N4830 containing pPX1601) elicits no protection. Although significant titers of anti-CS antibody are observed in these control groups, little protection against sporozoite challenge is seen.

Mice orally inoculated with 10^9 *S. dublin* (carrying pPX1601) or *S. dublin* control cells and boosted after 4 weeks with 10^{10} of those same organisms, were challenged at week 13. Eight days after challenge with 10^3 *P. berghei* sporozoites by tail vein injection, 4 out of 5 of the animals immunized with the control *S. dublin* cells exhibited patent blood stage parasitemia, whereas only 1 out of 5 of the animals immunized with *S. dublin* (carrying pPX1601) showed blood-stage parasites. Thus, 20% of the control animals escaped infection, whereas 80% of the immunized animals were protected.

Thus, attenuated live Salmonella are capable of delivering a sporozoite epitope to the immune system in such a manner as to elicit protective immunity not achieved by customary routs of vaccination.

9. DEPOSIT OF MICROORGANISMS

The following bacterial strains, carrying the listed plasmids encoding a Plasmodium epitope, have been deposited with the American Type Culture Collection (ATCC), Rockville, Md., and have been assigned the indicated accession numbers:

Bacterial Strain	Plasmid	Accession Number
Salmonella typhi Ty523	pPX1532. A plasmid which expresses a fusion protein of the first thirty amino acids of LT-B followed by a dimer of four tetrapeptide repeats, each of the *P. falciparum* CS protein, fused to 28 amino acids read out-of-frame from the LT-B gene. This fusion protein is expressed from the lac promoter.	67519
Salmonella enteritidis serotype dublin SL1438	pPX1528: A plasmid which expresses a fusion protein of the first thirty amino acids of LT-B at the amino-terminus followed by 223 amino acids of the *P. berghei* CS protein transcribed from the XmnI fragment of the CS protein gene, with 28 amino acids read out-of-frame from the LT-B gene at the carboxy-terminus. This fusion protein is expressed from the tac promoter.	67521
Salmonella enteritidis serotype dublin SL1438	pPX1601: A plasmid which expresses a fusion protein of the first thirty amino acids of LT-B at the amino-terminus followed by 223 amino acids of the *P. berghei* CS protein transcribed from the XmnI fragment of the CS protein gene, with 28 amino acids read out-of-frame from the LT-B gene at the carboxy-terminus. This fusion protein is expressed from the P_L promoter.	67520
E. coli N99c1	pPX1534: A plasmid which expresses the full-length *P. falciparum* CS protein gene (lacking only the sequence encoding the putative 16 amino acid signal sequence). This protein is expressed from the P_L promoter.	67518

The present invention is not to be limited in scope by the microorganisms deposited since the deposited embodiment is intended as a single illustration of one aspect of the invention and any microorganisms which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description, and figures which diagrammatically depict DNA sequences are not necessarily drawn to scale.

What is claimed is:

1. An attenuated enteroinvasive bacterium of the genus Salmonella or Shigella comprising a recombinant DNA sequence which encodes an epitope of the circumsporozoite protein of a malaria parasite.

2. The recombinant bacterium of claim 1 wherein the bacterium is *Salmonella typhi*, *Salmonella typhimurium* or *Salmonella enteritidis*.

3. The bacterium of claim 2 which is selected from the group consisting of Ty21, Ty21a, Ty523 and Ty541.

4. The recombinant bacterium of claim 2 wherein the bacterium is a serotype dublin.

5. The bacterium of claim 1 wherein the epitope of the repeat region of the circumsporozoite protein.

6. The bacterium of claim 1 wherein the epitope is Region I or Region II of the circumsporozoite protein.

7. The bacterium of claim 1 wherein the recombinant DNA sequence encodes a fusion protein comprising an epitope of the circumsporozoite protein and the B-subunit of the heat-labile enterotoxin of *E. coli* or a portion thereof, such that said fusion protein is immunogenic.

8. The bacterium of claim 1 wherein the DNA sequence is expressed under the control of the lac operon promoter of *E. coli*, the tac promoter, the leftward promoter of bacteriophage lambda, or the rightward promoter of bacteriophage lambda.

9. The bacterium of claim 1 or 2 wherein the malaria parasite is *Plasmodium falciparum*.

10. The bacterium of claim 9 wherein the epitope comprises the amino acid sequence asn-ala-asn-pro.

11. The bacterium of claim 1 wherein the bacterium is *Salmonella typhi* ATCC accession number 67519.

12. The bacterium of claim 1 wherein the malaria parasite is *Plasmodium vivax*.

13. The bacterium of claim 1 wherein the malaria parasite is *Plasmodium ovale*.

14. The bacterium of claim 1 wherein the malaria parasite is *Plasmodium malariae*.

15. The bacterium of claim 1 wherein the malaria parasite is *Plasmodium berghei*.

16. The bacterium of claim 15 wherein the epitope comprises the amino acid sequence asp-pro-ala-pro-pro-asn-ala-asn.

17. The bacterium of claim 1 wherein the bacterium is *Salmonella enteriditis* ATCC accession number 67521.

18. The bacterium of claim 1 wherein the bacterium is *Salmonella enteritidis* ATCC accession number 67520.

19. The bacterium of claim 1 wherein the malaria parasite is *Plasmodium yoelii*.

20. The bacterium of claim 1 wherein the malaria parasite is *Plasmodium knowlesi*.

21. The bacterium of claim 1 wherein the malaria parasite is *Plasmodium cynomolgi*.

22. A method of expressing an epitope of the circumsporozoite protein of a malaria parasite comprising:

a. transforming an attenuated enteroinvasive bacterium of the genus Salmonella or Shigella with a vector comprising a recombinant DNA sequence which encodes an epitope of the circumsporozoite protein of a malaria parasite; and

b. allowing the bacterium to grow under conditions which induce the expression of said circumsporozoite protein.

23. The method of according to claim 22 wherein the bacterium is Salmonella.

24. The method according to claim 23 wherein the bacterium is *Salmonella typhi*, *Salmonella typhimurium* or *Salmonella enteritidis*.

25. The method according to claim 22 wherein the DNA sequence is expressed under the control of the lac operon promoter of *E. coli*.

26. The method according to claim 23 wherein the DNA sequence is expressed under the control of the lac operon promoter of *E. coli*.

27. The method according to claim 26 wherein the bacterium is *Salmonella typhi* ATCC accession number 67519.

28. The method according to claim 22 wherein the DNA sequence is expressed under the control of the tac promoter.

29. The method according to claim 23 wherein the DNA sequence is expressed under the control of the tac promoter.

30. The method according to claim 29 wherein the bacterium is *Salmonella enteritidis* ATCC accession number 67521.

31. The method according to claim 22 wherein the DNA sequence is expressed under the control of the leftward promoter of bacteriophage lambda.

32. The method according to claim 23 wherein the DNA sequence is expressed under the control of the leftward promoter of bacteriophage lambda.

33. The method according to claim 32 wherein the bacterium is *Salmonella enteritidis* ATCC accession number 67520.

34. The method according to claim 22 or 23 wherein the DNA sequence is expressed under the control of the rightward promoter of bacteriophage lambda.

35. The method according to claim 22 or 23 wherein the malaria parasite is *Plasmodium falciparum*.

36. The method according to claim 22 wherein the malaria parasite is *Plasmodium vivax*.

37. The method according to claim 22 wherein the malaria parasite is *Plasmodium ovale*.

38. The method according to claim 22 wherein the malaria parasite is *Plasmodium malariae*.

39. The method according to claim 22 or 23 wherein the malaria parasite is *Plasmodium berghei*.

40. The method according to claim 22 wherein the malaria parasite is *Plasmodium yoelii*.

41. The method according to claim 22 wherein the malaria parasite is *Plasmodium knowlesi*.

42. The method according to claim 22 wherein the malaria parasite is *Plasmodium cynomolgi*.

43. An attenuated enteroinvasive bacterium of the genus Salmonella or Shigella having an arcA or galE mutation and comprising a recombinant DNA sequence which encodes an epitope of the circumsporozoite protein of a malaria parasite.

44. The attenuated bacterium of claim 43, wherein the circumsporozoite protein is derived from *Plasmodium falciparum*.

45. The attenuated bacterium of claim 43, wherein the malaria parasite is *Plasmodium berghei*.

46. The attenuated bacterium of claim 43, wherein the recombinant DNA sequence encodes a fusion protein comprising an epitope of the circumsporozoite protein and the B-subunit of heat-labile enterotoxin of *E. coli* or a portion thereof, such that said fusion protein is immunogenic.

47. The attenuated bacterium of claim 46, wherein the fusion protein comprises the N-terminal 30 amino acids of the B subunit of the heat-labile enterotoxin of *E. coli*.

48. The attenuated bacterium of claim 46, wherein the bacterium is *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella dublin* or *Salmonella enteritidis*.

49. The attenuated bacterium of claim 46, wherein the malaria parasite is *Plasmodium falciparum* or *Plasmodium vivax*.

50. The attenuated bacterium of claim 46, wherein the malaria parasite is *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium berghei*, *Plasmodium yoelii*, *Plasmodium knowlesi* or *Plasmodium cynomolgi*.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,112,749

DATED : May 12, 1992

INVENTOR(S) : Brey, III et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 41, Claim 5, line 5, delete "of" (2nd occ.) and insert ---is---.

Column 42, Claim 43, line 41, delete "arcA" and insert ---aroA---.

Signed and Sealed this

Seventh Day of September, 1993

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

US005348867A

United States Patent [19]

Georgiou et al.

[11] Patent Number: 5,348,867

[45] Date of Patent: Sep. 20, 1994

[54] **EXPRESSION OF PROTEINS ON BACTERIAL SURFACE**

[75] Inventors: George Georgiou, 11501 Juniper Ridge Dr., Austin, Tex. 78759; Joseph A. Francisco; Charles F. Earhart, both of Austin, Tex.

[73] Assignee: George Georgiou, Austin, Tex.

[21] Appl. No.: 794,731

[22] Filed: Nov. 15, 1991

[51] Int. Cl.[5] C12P 21/06; C12P 21/04; C12N 15/00; C12N 1/20

[52] U.S. Cl. 435/69.7; 435/69.8; 435/71.1; 435/71.2; 435/172.1; 435/252.1; 435/252.3; 435/252.33; 435/320.1; 536/23.4

[58] Field of Search 536/27; 435/69.7, 69.8, 435/71.1, 71.2, 172.1, 252.1, 252.3, 252.33, 320.1

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(List continued on next page.)

Primary Examiner—Robert J. Hill, Jr.
Assistant Examiner—Marianne Porta Allen
Attorney, Agent, or Firm—Arnold, White & Durkee

[57] ABSTRACT

The invention relates to a method for producing stable, surface-expressed polypeptides from recombinant gram-negative bacterial cell hosts. A tripartite chimetic gene and its related recombinant vector include separate DNA sequences for directing or targeting and translocating a desired gene product from a cell periplasm to the external cell surface. A wide range of polypeptides may be efficiently surface expressed, including β-lactamase and alkaline phosphatase. Full enzyme activity is maintained and the proteins remain anchored to the bacterial outer membrane surface.

19 Claims, 10 Drawing Sheets

lpp-ompA-X FUSIONS

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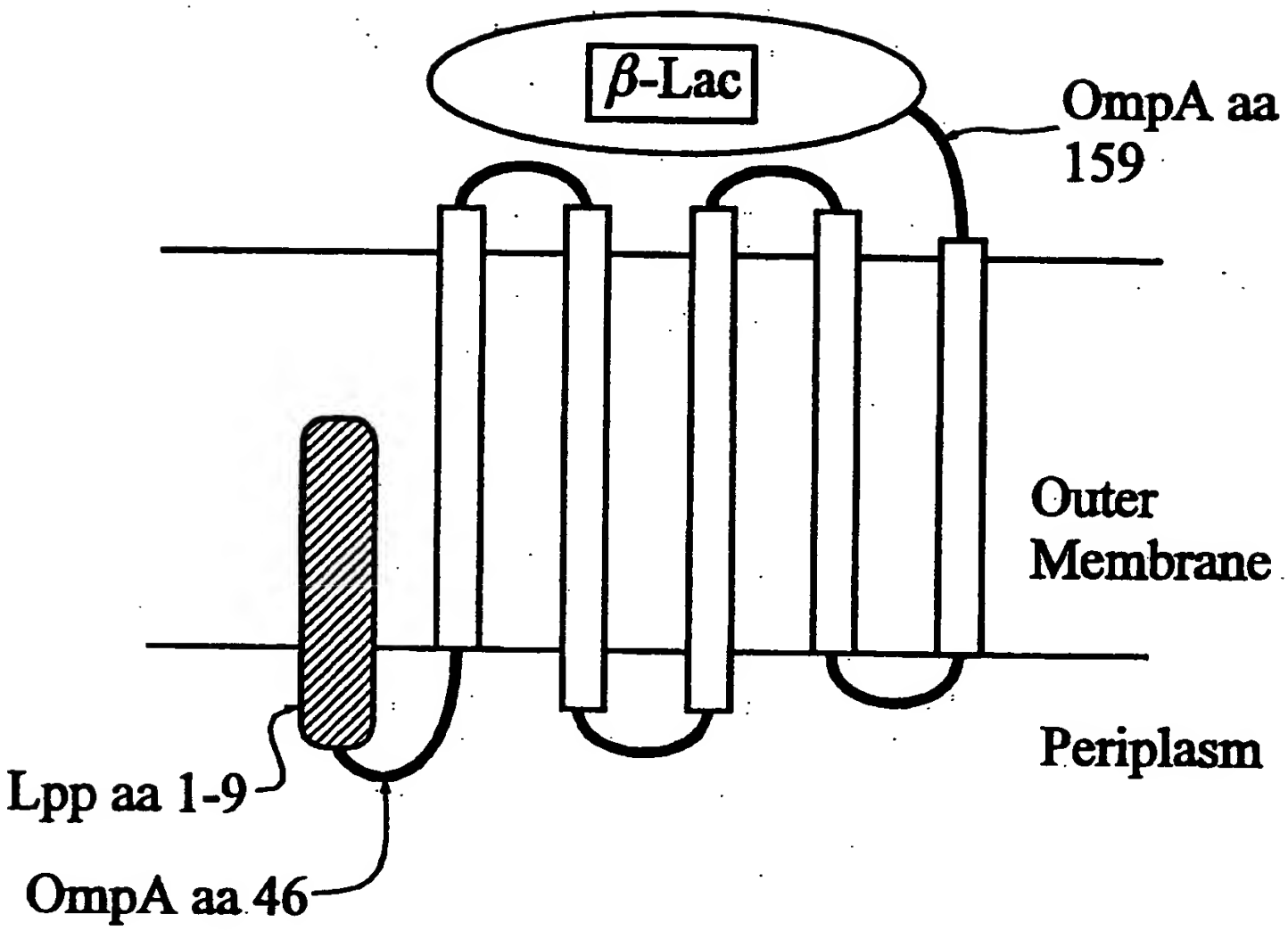
β-Lac
OmpA aa 159
Outer Membrane
Periplasm
Lpp aa 1-9
OmpA aa 46

FIGURE 1

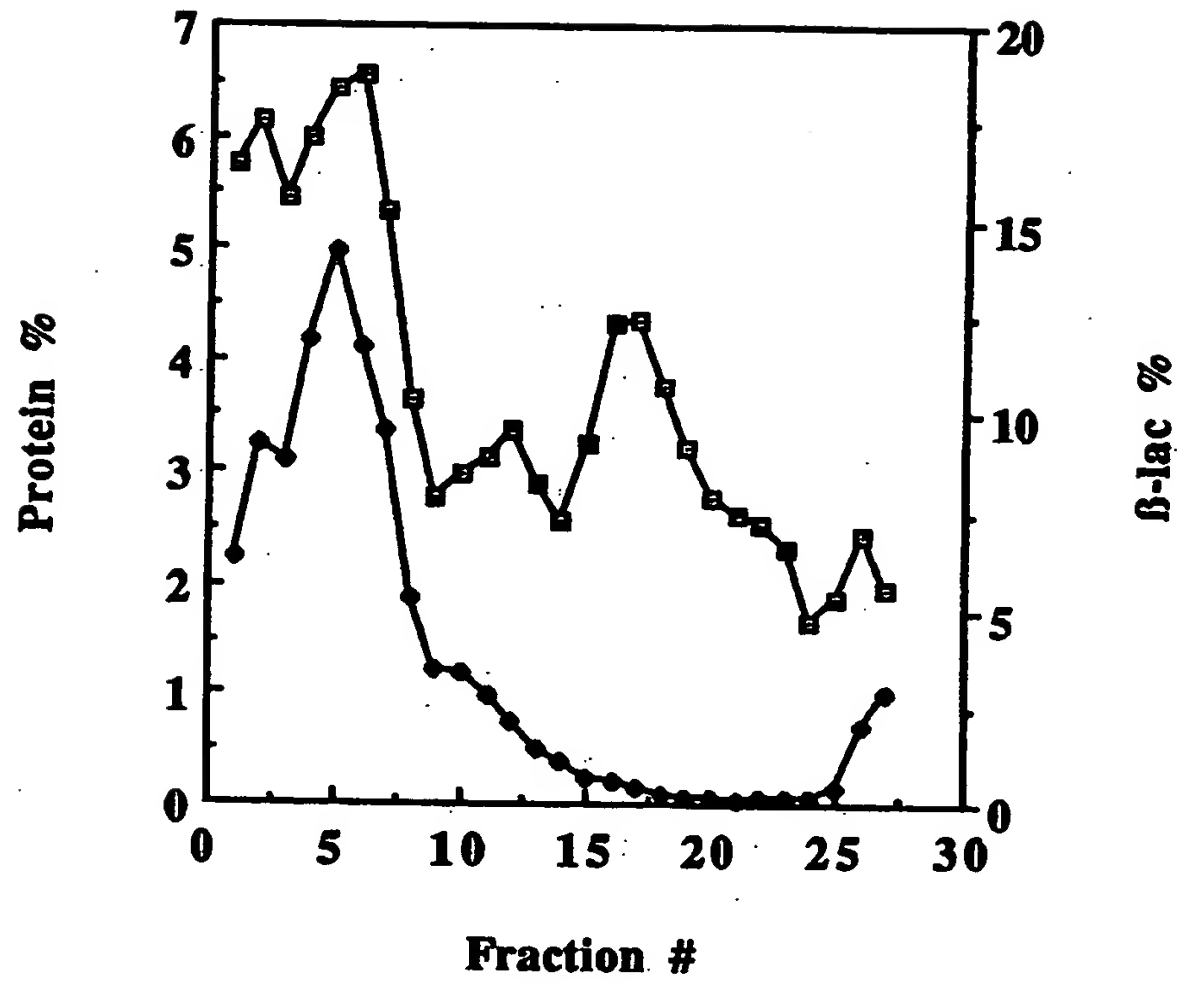

FIGURE 2

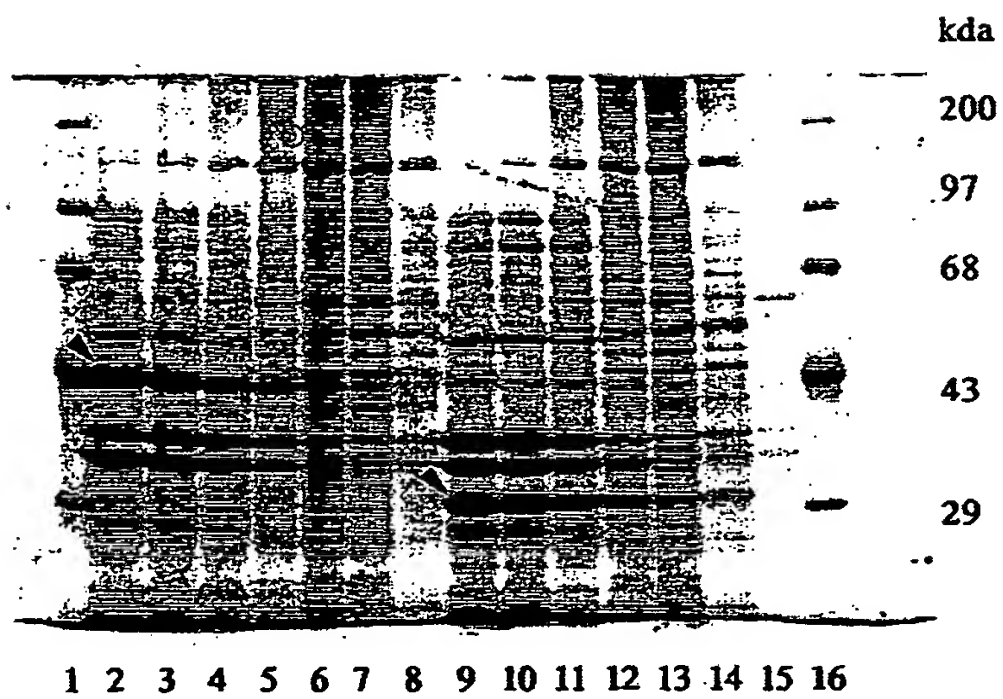

FIGURE 3A

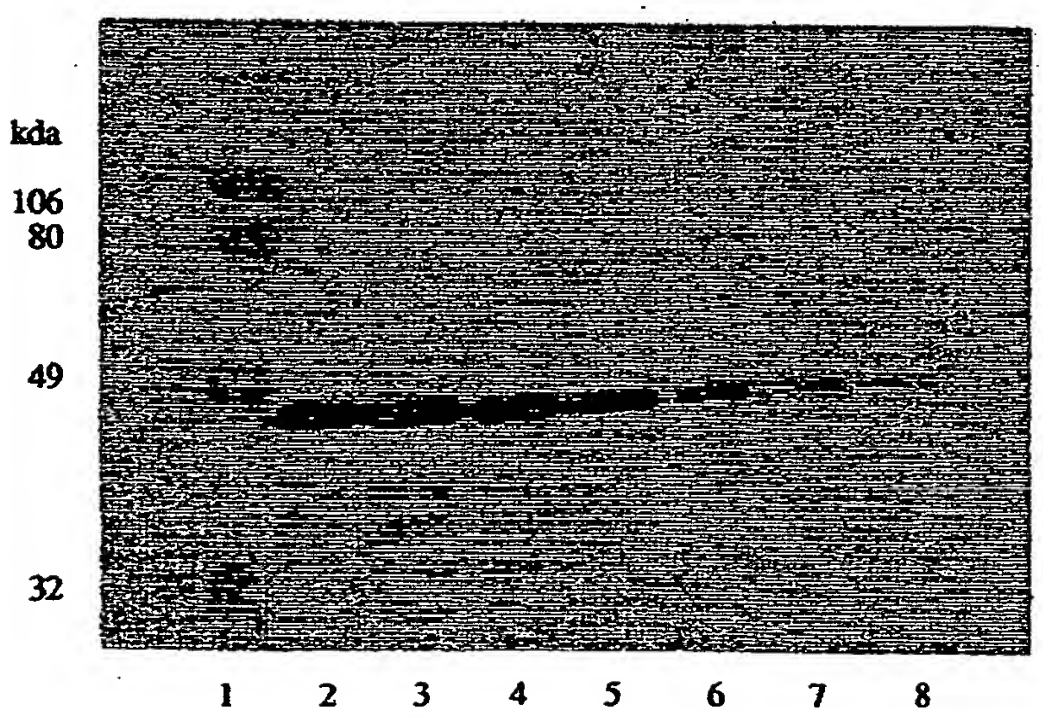

FIGURE 3B

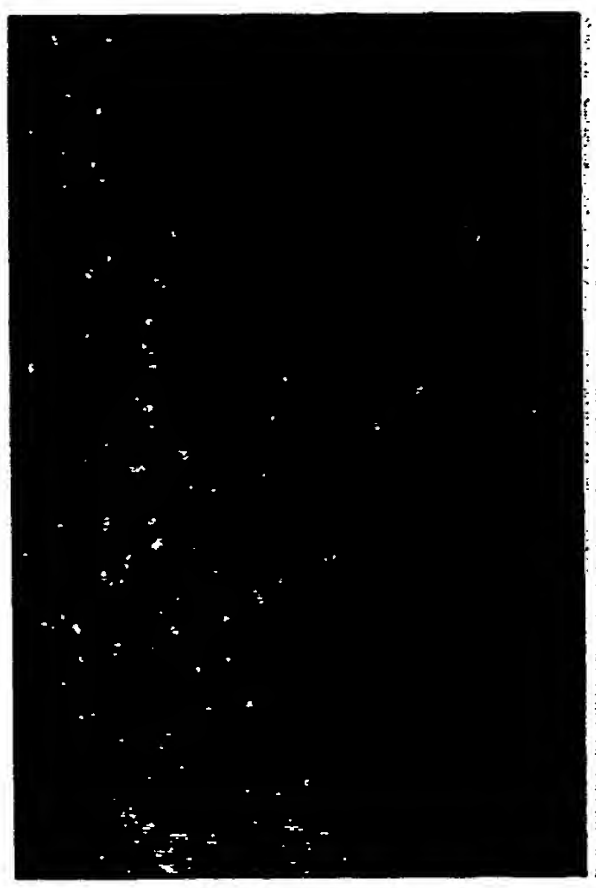

FIGURE 4A

FIGURE 4B

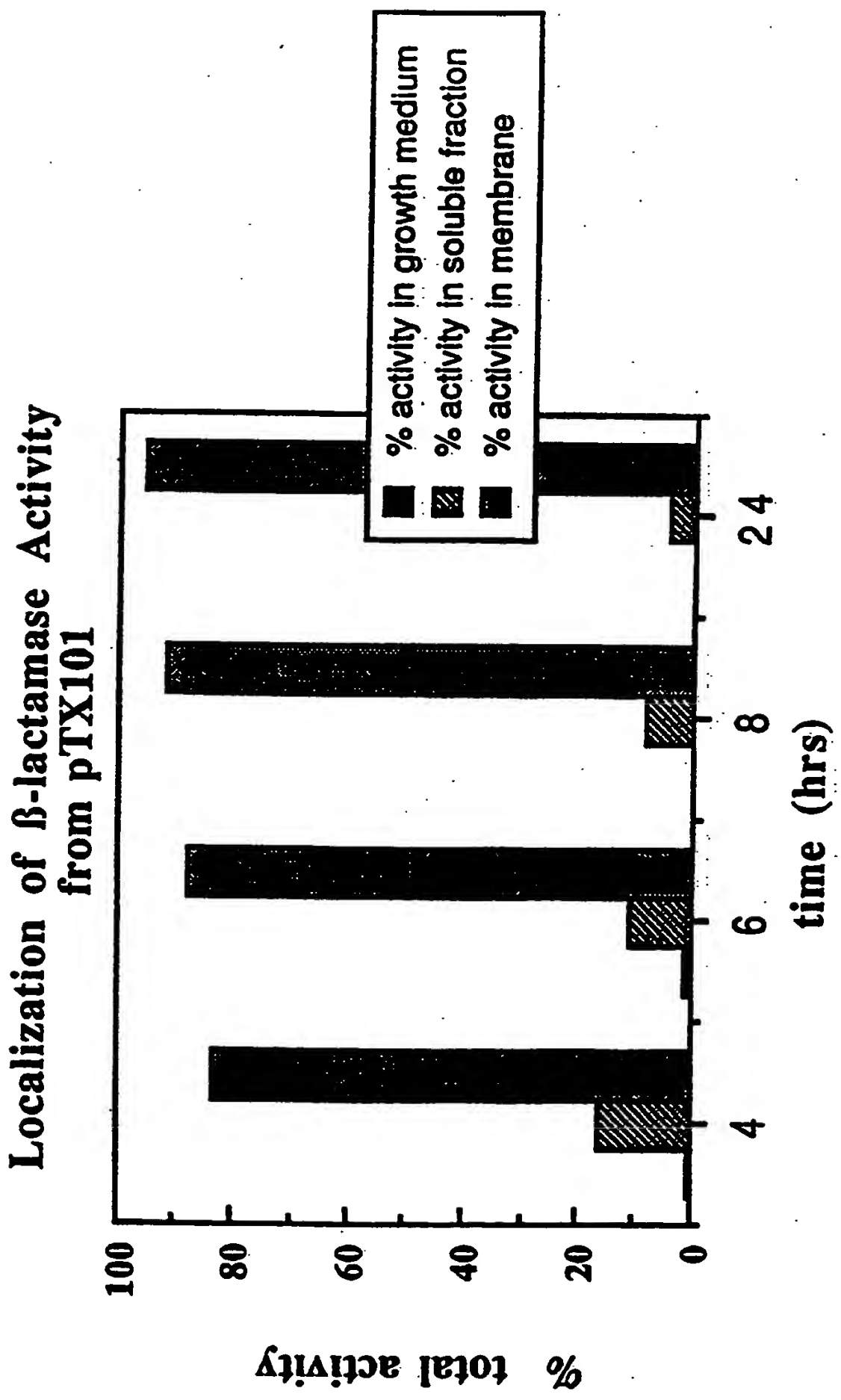
Localization of ß-lactamase Activity from pTX101
% activity in growth medium
% activity in soluble fraction
% activity in membrane
% total activity
0
20
40
60
80
100
4
6
8
24
time (hrs)

FIGURE 5

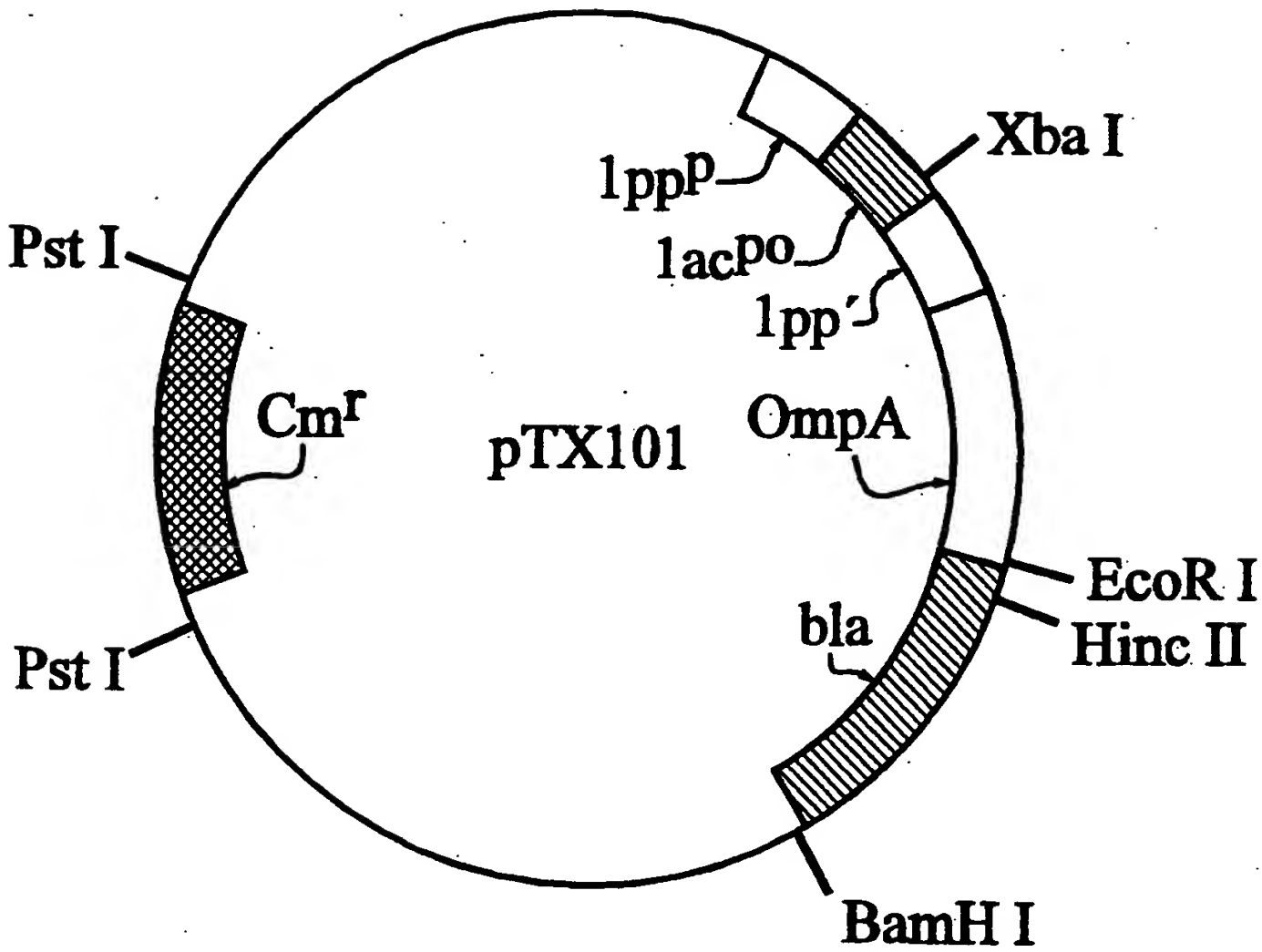
Xba I
lpp^p
lac^po
lpp'
Pst I
Cm^r
pTX101
OmpA
EcoR I
Hinc II
bla
Pst I
BamH I

FIGURE 6

```
ATGAAAGCTA CTAAACTGGT ACTGGGCGCG GTAATCCTGG GTTCTACTCT 50
GCTGGCAGGT TGCTCCAGCA ACGCTAAAAT CGATCAGGGA ATTAACCCGT 100
ATGTTGGCTT TGAAATGGGT TACGACTGGT TAGGTCGTAT GCCGTACAAA 150
GGCAGCGTTG AAAACGGTGC ATACAAAGCT CAGGGCGTTC AACTGACCGC 200
TAAACTGGGT TACCCAATCA CTGACGACCT GGACATCTAC ACTCGTCTGG 250
GTGGCATGGT ATGGCGTGCA GACACTAAAT CCAACGTTTA TGGTAAAAAC 300
CACGACACCG GCGTTTCTCC GGTCTTCGCT GGCGGTGTTG AGTACGCGAT 350
CACTCCTGAA ATCGCTACCC GTCTGGAATA CCAGTGGACC AACAACATCG 400
GTGACGCACA CACCATCGGC ACTCGTCCGG ACAACGGAAT TCCGGGTCAC 450
CCAGAAACGC TGGTGAAAGT AAAAGATGCT GAAGATCAGT TGGGTGCACG 500
AGTGGGTTAC ATCGAACTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT 550
TTCGCCCCGA AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCTGCTA 600
TGTGGCGCGG TATTATCCCG TGTTGACGCC GGGCAAGAGC AACTCGGTCG 650
CCGCATACAC TATTCTCAGA ATGACTTGGT TGAGTACTCA CCAGTCACAG 700
AAAAGCATCT TACGGATGGC ATGACAGTAA GAGAATTATG CAGTGCTGCC 750
ATAACCATGA GTGATAACAC TGCGGCCAAC TTACTTCTGA CAACGATCGG 800
AGGACCGAAG GAGCTAACCG CTTTTTTGCA CAACATGGGG GATCATGTAA 850
CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC 900
GAGCGTGACA CCACGATGCC TGCAGCAATG GCAACAACGT TGCGCAAACT 950
ATTAACTGGC GAACTACTTA CTCTAGCTTC CCGGCAACAA TTAATAGACT 1000
GGATGGAGGC GGATAAAGTT GCAGGACCAC TTCTGCGCTC GGCCCTTCCG 1050
GCTGGCTGGT TTATTCGTGA TAAATCTGGA GCCGGTGAGC GTGGGTCTCG 1100
CGGTATCATT GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG 1150
TTATCTACAC GACGGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG 1200
ATCGCTGAGA TAGGTGCCTC ACTGATTAAG CATTGGTAAC TGTCAGACCA 1250
AGTTTACTCA TATATACTTT AGA                                1273
```

FIGURE 7

Lpp
ATG AAA GCT ACT AAA CTG GTA CTG GGC GCG GTA ATC CTG GGT TCT
Met Lys Ala Thr Lys Leu Val Leu Gly Ala Val Ile Leu Gly Ser

ACT CTG CTG GCA GGT *TGC TCC AGC AAC GCT AAA ATC GAT CAG
Thr Leu Leu Ala Gly Cys Ser Ser Asn Ala Lys Ile Asp Gln

Linker
GGA ATT
Gly Ile

OmpA aa 46-159
AAC CCG TAT GTT GGC TTT GAA ATG GGT TAC GAC TGG TTA GGT CGT
Asn Pro Tyr Val Gly Phe Glu Met Gly Tyr Asp Trp Leu Gly Arg

ATG CCG TAC AAA GGC AGC GTT GAA AAC GGT GCA TAC AAA GCT CAG
Met Pro Tyr Lys Gly Ser Val Glu Asn Gly Ala Tyr Lys Ala Gln

GGC GTT CAA CTG ACC GCT AAA CTG GGT TAC CCA ATC ACT GAC GAC
Gly Val Gln Leu Thr Ala Lys Leu Gly Tyr Pro Ile Thr Asp Asp

CTG GAC ATC TAC ACT CGT CTG GGT GGC ATG GTA TGG CGT GCA GAC
Leu Asp Ile Tyr Thr Arg Leu Gly Gly Met Val Trp Arg Ala Asp

ACT AAA TCC AAC GTT TAT GGT AAA AAC CAC GAC ACC GGC GTT TCT
Thr Lys Ser Asn Val Tyr Gly Lys Asn His Asp Thr Gly Val Ser

CCG GTC TTC GCT GGC GGT GTT GAG TAC GCG ATC ACT CCT GAA ATC
Pro Val Phe Ala Gly Gly Val Glu Tyr Ala Ile Thr Pro Glu Ile

GCT ACC CGT CTG GAA TAC CAG TGG ACC AAC AAC ATC GGT GAC GCA
Ala Thr Arg Leu Glu Tyr Gln Trp Thr Asn Asn Ile Gly Asp Ala

CAC ACC ATC GGC ACT CGT CCG GAC AAC
His Thr Ile Gly Thr Arg Pro Asp Asn

Linker
GGA ATT CCG GGT
Gly Ile Pro Gly

ß-lactamase
CAC CCA GAA ACG CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG
His Pro Glu Thr Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu

GGT GCA CGA GTG GGT TAC ATC GAA CTG GAT CTC AAC AGC GGT AAG
Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp Leu Asn Ser Gly Lys

ATC CTT GAG AGT TTT CGC CCC GAA GAA CGT TTT CCA ATG ATG AGC
Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe Pro Met Met Ser

ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA TTA TCC CGT GTT GAC
Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser Arg Val Asp

FIGURE 8

```
GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT TCT CAG AAT
Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser Gln Asn

GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG GAT
Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr Asp

GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT
Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser

GAT AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA CCG
Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro

AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT
Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr

CGC CTT GAT CGT TGG GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC
Arg Leu Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn

GAC GAG CGT GAC ACC ACG ATG CCT GCA GCA ATG GCA ACA ACG TTG
Asp Glu Arg Asp Thr Thr Met Pro Ala Ala Met Ala Thr Thr Leu

CGC AAA CTA TTA ACT GGC GAA CTA CTT ACT CTA GCT TCC CGG CAA
Arg Lys Leu Leu Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln

CAA TTA ATA GAC TGG ATG GAG GCG GAT AAA GTT GCA GGA CCA CTT
Gln Leu Ile Asp Trp Met Glu Ala Asp Lys Val Ala Gly Pro Leu

CTG CGC TCG GCC CTT CCG GCT GGC TGG TTT ATT CGT GAT AAA TCT
Leu Arg Ser Ala Leu Pro Ala Gly Trp Phe Ile Ala Asp Lys Ser

GGA GCC GGT GAG CGT GGG TCT CGC GGT ATC ATT GCA GCA CTG GGG
Gly Ala Gly Glu Arg Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly

CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC TAC ACG ACG GGG
Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile Tyr Thr Thr Gly

AGT CAG GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT GAG ATA
Ser Gln Ala Thr Met Asp Glu Arg Asn Arg Gln Ile Ala Glu Ile

GGT GCC TCA CTG ATT AAG CAT TGG*TAA CTG TCA GAC CAA GTT TAC
Gly Ala Ser Leu Ile Lys His Trp

TCA TAT ATA CTT TAG A
```

FIGURE 8 (CONT.)

FIGURE 9

EXPRESSION OF PROTEINS ON BACTERIAL SURFACE

The United States Government may have certain rights in the present invention pursuant to Grant No. BCS-9013007 awarded by the National Science Foundation.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates generally to the exportation and localization of polypeptides to the external membrane surface of a gram-negative cell, to recombinant vectors useful for the transformation of a host cell and to chimeric genes that provide outer membrane targeting and transmembrane sequences. Methods are disclosed providing for surface expression of proteins, including antigenically active proteins, specific binding proteins and enzymatically active species.

2. Description of Related Art

There is substantial interest in the expression of selected proteins on the surface of bacteria. Many potential applications exist, including the production of genetically engineered whole cell adsorbents, construction of "peptide libraries" where bacteria carry different exposed sequences, cell-bound enzymes (another form of immobilization), and use as live vaccines or immunogens to generate antibodies.

One approach to obtaining surface expressed foreign proteins has been to use a native membrane protein as a carrier for a foreign protein. LamB, an outer membrane protein of *Escherichia coli*, has been fused with peptides varying in length up to about 60 amino acids with successful expression of the hybrid protein at a recombinant host cell surface (Charbit, et al., 1991). Unfortunately, only relatively short polypeptides are surface-expressed using this method. Outer membrane proteins have "loop" regions spanning the membrane surface and while it is possible to substitute foreign DNA into the gene regions encoding the loop regions, there are only a limited number of insertions possible, constrained by the size of the loop region and, apparently, by the requirement to preserve the penetration and translocating properties of the membrane protein.

In general, attempts to develop methods of anchoring larger proteins as well as the smaller peptides on a bacterial cell surface have focused on fusion of the desired recombinant polypeptide to a native protein that is normally exposed on the cell's exterior in hope that the resulting hybrid will also be localized on the surface. The problem with this approach is that fusion of the foreign protein interferes with localization and, in many cases, the hybrid molecule is unable to reach the cell surface.

Nevertheless, in one example employing the Klebsiella enzyme pullulanase, a normally periplasmic protein, β-lactamase, was translocated through the *E. coli* outer membrane. C-terminal regions of pullulanase were replaced with DNA segments encoding β-lactamase or alkaline phosphatase. Only the hybrid protein with β-lactamase was transported to the cell surface (Kornacker and Pugsley, 1990). However, the surface-expressed protein was only transiently anchored to the cell surface, suggesting a severe limitation on the potential value of any other proteins expressed by this method as surface immunogens, adsorbents, or surface immobilized species. Furthermore, the assembly of pullulanase fusions onto the cell surface is a very complicated process requiring the presence of at least 14 foreign gene products in the host cell. It should be noted that alkaline phosphatase fused to the same pullulanase sequence could not be localized on the cell surface (Kornacker and Pugsley 1990).

The mechanisms of protein insertion within- and translocation across- the outer membrane of gram-negative bacteria are not well understood. For some outer membrane proteins, such as the PhoE porin, the information necessary for proper localization and assembly is interspersed within the primary sequence (Bosch et al., 1986; Bosch et al., 1989). Alternatively, the targeting signal may be contained within a single short continuous segment. For example, the first nine N-terminal amino acids of the major *E. coli* lipoprotein are necessary for proper localization in the outer membrane. Fusion to this short sequence is sufficient to direct the normally soluble periplasmic protein β-lactamase to the outer membrane (Ghrayeb and Inouye, 1984). Similarly, extensive studies with OmpA have suggested that the region between residues 154 and 180 is crucial for localization (Klose et al., 1988a, 1988b). With OmpA, targeting and outer membrane assembly appear to be distinct events. Only large fragments containing the entire membrane spanning sequence of OmpA are able to assemble into a conformation exhibiting native resistance to proteolytic digestion (Klose et al., 1988a).

In general, amino acid substitutions or insertions within outer membrane loops exposed on the cell surface are well tolerated and do not interfere with the folding of the protein in the membrane. Peptides as large as 60 amino acids have been inserted within external loops of various outer membrane proteins and appear to be exposed on the surface of intact *E. coli* cells as indicated by immunochemical techniques (Charbit et al., 1991). However, efforts to direct soluble reporter proteins such as alkaline phosphatase, to the cell surface using outer membrane protein fragments have not been successful. These fusions either end up at incorrect cellular locations or become anchored in the membrane with the secreted protein domain facing the periplasm (Murphy et al., 1990). In gram-negative bacteria the outer membrane acts as a barrier to restrict the export of proteins from the cell. Normally only pilins, flagellins, specific enzymes and a few toxins are completely transported across the outer membrane (Kornacker and Pugsley, 1990). Most of these proteins are first secreted into the periplasmic space via the general secretion pathway and then cross the outer membrane by a process that involves the action of several additional gene products (Filloux et al., 1990).

Whole cell adsorbents are considered to have potential value for biotechnology applications for the purification of various molecules or the selective removal of hazardous compounds from contaminated waste waters. However, a major constraint in the development of whole cell adsorbents is the availability of bacterial strains with suitable ligands on their surface. Although functional antibody fragments have been produced in *Escherichia coli* (Skerra and Pluckthun 1988, Better et al. 1988, Orlandi et al. 1989, Sastry et al. 1989), these polypeptides have not been expressed on the cell surface. Indeed, a "library" of recombinant immunoglobulins containing both heavy and light variable domains (Huse et al. 1989) has been produced with the proteins having antigen-binding affinity comparable to the corresponding natural antibodies. Furthermore, the variety

of recombinant immunoglobulins from bacteria is greater than the number of antibody molecules that can be generated by the mammalian cell. In this way it has become possible to expand the repertoire of antibodies that can be made by the immune system (Huse et al. 1989). While the availability of such a wide range of immunoglobulins suggests the potential for creation of *E. coli* cells endowed with immunological surface receptors, there has been little success in producing recombinant proteins on the surface of bacterial cells, and conspicuous lack of a method to generate recombinant immunoglobulins on surfaces of gram negative host cells.

Although the potential repertoire of immunoglobulins produced in an immunized animal is high ($>10^{10}$), only a small number of monoclonal antibodies can be generated using hybridomas. This limitation complicates the isolation of antibodies with specific properties, such as the ability to act as a catalyst. Combinational antibody libraries comprising millions of genes of different antibodies have been cloned using phage λ(Huse et al., 1989). However, screening the library to select the desired clone can be extremely time consuming and complicated. One approach to the screening problem has been an attempt to express antibodies on the surface of filamentous phage. Phage particles displaying high affinity antibody molecules on their surface can be enriched by chromatography through a column of immobilized antigen (Barbas et al., 1991; Clarckson et al., 1991; Breitling, 1991). Although the feasibility of this technique has been demonstrated, several problems are apparent, including: (1) fusion to bacteriophage coat proteins causing interference with antibody folding, (2) subcloning of large numbers of positive phage particles in order to produce soluble antibody fragments to carry out more extensive characterization, and (3) lack of control of the number of antibody molecules on the phage surface, thus affecting binding to the immobilized antigen and complicating the selection procedure.

SUMMARY OF THE INVENTION

The present invention addresses one or more of the foregoing problems in providing a versatile recombinant vector that will promote transport of a periplasmic or other protein to the external face of the outer membrane of a gram-negative bacterial cell in the absence of any specific export components. In particular, the vector includes a tripartite chimeric gene having a membrane targeting sequence, a membrane translocating sequence capable of locating a fusion protein on the outer surface and a gene segment encoding any of a variety of proteins.

Overall and in general the tripartite chimeric genes of the invention include at least three DNA segments. One segment is a targeting DNA sequence encoding a polypeptide capable of targeting and anchoring the fusion polypeptide to a host cell outer membrane. Targeting sequences are well known and have been identified in several of membrane proteins including Lpp. Generally, as in the case of Lpp, the protein domains serving as localization signals are relatively short. The Lpp targeting sequence includes the signal sequence and the first 9 amino acids of the mature protein. These amino acids are found at the amino terminus of Lpp. *E. coli* outer membrane lipoproteins from which targeting sequences may be derived include TraT, OsmB, NlpB and BlaZ. Lipoprotein 1 from *Pseudomonas aeruginosa* or the PA1 and PCN proteins from Haemophilus influenza as well as the 17 kDa lipoprotein from *Rickettsia rickettsii* and the H.8 protein from *Neisseria gonorrhea* and the like may be used.

A second component of tripartite chimeric genes is a DNA segment encoding a membrane-transversing amino acid sequence. Transversing is intended to denote an amino acid sequence capable of transporting a heterologous or homologous polypeptide through the outer membrane. In preferred embodiments, the membrane transversing sequence will direct the fusion polypeptide to the external surface. As with targeting DNA segments, transmembrane segments are typically found in outer membrane proteins of all species of gram-negative bacteria. Transmembrane proteins, however, serve a different function from that of targeting sequences and generally include amino acids sequences longer than the polypeptide sequences effective in targeting proteins to the bacterial outer membrane. For example, amino acids 46–159 of the *E. coli* outer membrane protein OmpA effectively localize a fused polypeptide to the external surface of the outer membrane when also fused to a membrane targeting sequence. These surface exposed polypeptides are not limited to relatively short amino acid sequences as when they are incorporated into the loop regions of a complete transmembrane lipoprotein. While the invention has been demonstrated with a transmembrane directing protein sequence from OmpA, other transmembrane directing sequences from outer membrane proteins may be employed.

The third gene segment comprising the tripartite chimeric gene fusion is a DNA segment that encodes any one of a variety of desired polypeptides. This DNA segment is positioned downstream from the DNA segment encoding the transmembrane sequence. The tripartite chimeric gene when provided with a functional promoter is expressible in gram-negative host cells.

A particular embodiment of the invention includes recombinant vectors prepared from the herein-described tripartite chimeric gene fusions. Such vectors will express fusion polypeptides at the outer membrane cell surface of a gram-negative host cell. These recombinant vectors include a functional promoter sequence and a targeting DNA sequence encoding a protein capable of targeting to the outer surface of a gram-negative bacterial host cell. The targeting gene is typically positioned downstream of the promoter sequence. A transmembrane gene sequence is positioned downstream of the targeting gene sequence. The transmembrane sequence will encode a protein domain capable of transversing the cell outer membrane. The vector will also include a DNA sequence which encodes a desired protein. This sequence when positioned downstream of the transmembrane sequence will be expressed on the external surface of the outer membrane, and typically is exposed to the external medium while remaining stably anchored to the membrane surface.

A most preferred embodiment of the recombinant vector is plasmid pTX101. This plasmid contains a fusion of the signal sequence and the first 9 amino acids of the major outer membrane lipoprotein of *E. coli*, a 342-base pair fragment from the outer membrane protein OmpA and the coding sequence for the complete mature β-lactamase protein. However, clearly numerous variations of the disclosed recombinant vectors could be prepared using techniques well known to those of skill in the art. DNA sequences encoding regions from a wide variety of membrane proteins could be em-

ployed. Such regions may be fused with any of a number of genes or gene fragments via a polylinker region.

The polypeptides encoded by the nucleic acid segments identified herein have been described in terms of function related to targeting and transversing fusion polypeptides to a gram-negative bacterial cell outer membrane surface. The invention is intended to include variations of the fused genes disclosed to the extent that the encoded polypeptides are functionally biologically equivalent. In general, by biologically functionally equivalent is meant amino acid sequences that may vary from certain of the disclosed fusion products, by e.g., natural genetic drift, strain or subspecies antigenic variation or by mutation of the DNA molecules without loss of appropriate membrane targeting or transversing functions as described.

Likewise, certain changes in nucleic acid composition of genes encoding polypeptides having the aforementioned functions, will not affect the general broad concept of the invention. For example, vectors containing variant codons for a particular amino acid, while altering the DNA composition, will not change the amino acid identity. Minor base pair changes, while producing some variation in primary amino acid sequence of the encoded polypeptide, are not expected to substantially alter function. All such variations, whether in amino acid or nucleic acid composition, are contemplated to be within the scope of the invention.

The methods illustrated for the expression of desired recombinant polypeptides on the cell surface may also be achieved by fusion to protein domains other than those derived from the major lipoprotein and OmpA, provided that these domains can function for the expression of the desired polypeptide on the cell surface. Generally, the desired polypeptide is fused to an amino acid sequence that includes the signals for localization to the outer membrane and for translocation across the outer membrane. The amino acid sequences responsible for localization and for translocation across the outer membrane may be derived either from the same bacterial protein or from different proteins of the same or different bacterial species. Examples of proteins that may serve as sources of localization signal domains include *E. coli* outer membrane lipoproteins from such as TraT, OsmB, NlpB, BlaZ, *Pseudomonas aeruginosa* lipoprotein 1, *Haemophilus influenza PA*1 and PCN proteins, *Rickettsia rickettsii* 17 kDa lipoprotein, *Neisseria gonorrhea* H.8 protein and the like. A sequence that spans the outer membrane and serves to transport the desired recombinant polypeptide to the cell surface can be derived from a membrane spanning domain of suitable length from any native outer membrane protein of gram-negative bacteria, including the porins LamB, PhoE, OmpC and OmpF, as well as other outer membrane proteins such as OmpT, FepA, and the like.

Any of a wide variety of gram-negative bacteria may be useful in practicing the invention. Such gram-negative bacteria include *E. coli*, Salmonella, Klebsiella, Erwinia, and the like. *E. coli* and Salmonella are particularly preferred as host cells. Although there are variations among the bacteria outer membrane proteins are similar. Target and transversing sequences from any of the membrane proteins may be used in constructing vectors useful for exportation across the cell wall of gram-negative bacteria.

Another aspect of the invention includes transformants. A typical transformant is a Salmonella prepared by transformation with the described recombinant vectors. A most preferred transformant is *E. coli*.

The invention is typically practiced using one or more of the commonly available gram-negative bacteria as cell hosts. However, rough mutants having somewhat differing membrane compositions are expected to also be useful in the practice of the invention. Membranes with higher phospholipid content, for example, may for some fusion polypeptides, provide more efficient surface expression at higher temperatures. Alternatively, it may be desirable to anchor some polypeptides closer to the membrane surface with increased lipid-protein interactions, perhaps for the purpose of increasing immunogenic response or altering adsorbent properties. Such mutants, spontaneously generated or otherwise, are contemplated as useful as host cells and/or as sources for membrane directing and transversing sequences.

Numerous types of fusion polypeptides may be expressed using the aforementioned system. Relatively large proteins such as alkaline phosphatase have been expressed on the surface of *E. coli* host cells. In its dimeric form alkaline phosphatase has a molecular weight of greater than 80 kDa. Other large proteins are also expected to be effectively surface expressed. Examples of expressed polypeptides include β-lactamase, alkaline phosphatase, cellulose binding domain of cellulase, or single-chain F_v antibody.

Expression of a variety of single-chain antibodies on the surface of a gram-negative bacterial host cell has several potential important applications particularly for the preparation of whole cell adsorbents. In addition, a variety of antigenic determinants may be expressed on a cell surface and used to prepare bacterial vaccines. A selected antigen in combination with an activating agent such as IL-4 on the surface of a bacterium may have potential use in stimulating an immune response toward a surface exposed antigen.

Tripartite chimeric gene fusions or the recombinant vectors herein described will typically include appropriate promoters. Such promoters are well known to those of skill in the art and examples include lpp promoter or lac promoter. Additionally, recombinant vectors also include a signal peptide. In preferred embodiments the signal peptide is positioned upstream of the targeting gene segment in recombinant vectors.

The invention also includes a method for expressing a fusion polypeptide anchored on the outer membrane surface of a gram-negative bacterial host cell. A gene segment encoding a desired polypeptide is selected and inserted by the herein described methods into one or more of the disclosed recombinant vectors. A selected gram-negative cell is transformed with the vector. The transformants are cultured and screened in order to identify clone transformants having a desired peptide expressed on the host cell surface. There are numerous ways the desired gene segment encoding the polypeptide could be incorporated into one or more of the disclosed recombinant vectors. For example, plasmid pTX101 may be cut with the restriction endonuclease EcoRI at the unique site in the linker region between the OmpA and βlactamase sequence. Typically, blunt ends are created on the DNA by treating with the Klenow fragment of DNA polymerase. Plasmids containing the coding sequence for the desired polypeptide may be isolated and DNA fragments obtained by cutting that plasmid with an appropriate endonuclease followed by blunt ending again using a Klenow frag-

ment or similar polymerase. The linearized pTX101 vector and the desired gene fragment may then be ligated and the resultant DNA transformed into an appropriate bacterial host cell strain such as *E. coli* strain JM109.

Surprisingly, the temperature at which the cells are cultured has an effect on the expression of the desired polypeptide. Culturing at higher temperatures, about 40° C., for example, results in less efficient expression of the desired polypeptide on the surface of the bacterial host. Although expression on the surface may be obtained when culturing is performed between about 22°–40° C., a preferred temperature range is between 22°–27° C. and a most preferred temperature being around 24° C.

Yet another aspect of the present invention is a method for obtaining an immunogenic polypeptide. An immunogenic polypeptide to which it is desired to elicit an immune response is selected and then inserted into an appropriate recombinant vector prepared in accordance with the aforementioned procedures. Appropriate gram-negative cells are transformed and the culture screened for transformants. Transformants are then screened to determine the degree of immunogenicity and those that are highly immunogenic are used to obtain one or more antibodies. This method is particularly useful because it is known that surface expressed polypeptides typically elicit higher antigenic and immunogenic responses than those peptides that are not immobilized on a bacterial surface. Surface exposed immunogenic polypeptides may also be used to prepare vaccines, typically by mixing the cells in a pharmaceutically acceptable vehicle suitable for administration in mammals.

Antibodies can be equally well expressed on the surface of the cell. When such antibodies are expressed on cell surfaces those with high affinity for particular antigens may be selected. Variants of antibodies may be prepared and surface expressed and antibody-like sequences may be prepared and tested for affinity to the appropriate antigens.

In yet another aspect of the invention it is contemplated that kits useful for transforming gram-negative bacterial host cells may be prepared. Kits will include at least one recombinant vector prepared in accordance with the herein described invention in an appropriately compartmentalized container. A preferred recombinant vector is defined by SEQ ID NO:1.

The invention also includes a method for removing contaminants from fluids. In this method various receptor proteins expressed on the outer membranes of gram-negative bacteria may be used to selectively interact with a wide variety of undesirable compounds. Metallothionein, for example, binds with a wide variety of heavy metals including iron, cadmium, zinc, copper, vanadium, and similar metals. When bound to the surface of a gram-negative organism this protein is expected to efficiently remove heavy metals from aqueous samples.

Whole cell adsorbents, with surface expressed polypeptides, such as selected antibodies, may be used to remove biological contaminants, for example, bacterial endotoxin from water samples. The efficiency of such whole cell adsorbents may be increased by cross-linking the bacterial surface. This also may increase the stabilization of the cells against disruption. One method of stabilization involves the specific cross-linking of the cells through the lipopolysaccharide component of the surface. Thus the cells can be aggregated and stabilized without affecting the function of surface-expressed proteins. Other types of cell adsorbents are contemplated including the use of cellulose binding domains, starch binding domains, protein A, lectins, or protease receptors expressed on outer membrane bacterial cell surfaces.

Still further embodiments of the invention include immobilized enzyme systems. Any one of a wide variety of biocatalytically active polypeptides may be expressed on the surface of a bacterial cell using the disclosed methods. Advantages of having an enzyme expressed on the bacterial cell surface include increased accessibility to substrates, stability, and potentially increased lipid solubility. In a more particular embodiment, biocatalytically active polypeptides immobilized on host cell membranes without additional bacterial host cell components may be used in biphasic reaction systems. Enhanced lipid solubility of the immobilized enzymes enables catalyst substrate interaction in the lipophilic solvents with extraction of the water soluble products into the aqueous phase. Further contemplated embodiments in such an immobilized system include encapsulating immobilized enzymes on membrane surfaces within liposomes or similar vesicles.

As part of the invention, kits useful for the expression of fusion proteins are also envisioned comprising a container having suitably aliquoted reagents for performing the foregoing methods. For example, the containers may include one or more vectors, examples being the vectors of claim 2, particular embodiments of which are shown schematically in FIG. 5. Suitable containers might be vials made of plastic or glass, various tubes such as test tubes, metal cylinders, ceramic cups or the like. Containers may be prepared with a wide range of suitable aliquots, depending on applications and on the scale of the preparation. Generally this will be an amount that is conveniently handled so as to minimize handling and subsequent volumetric manipulations. Most practitioners will prefer to select suitable nucleases such as EcoRI, BamHI, or PstI from common supplies usually on hand; however, such restriction endonucleases could also be optionally included in a kit preparation.

Vectors supplied in kit form are preferably supplied in lyophilized form, although such DNA fragments may also be taken up in a suitable solvent such as ethanol, glycols or the like and supplied as suspensions. For most applications, it would be desirable to remove the solvent which for ethanol, for example, is a relatively simple matter of evaporation.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram of the Lpp-OmpA-β-lactamase fusion in the outer membrane of a gram negative bacterium. Rectangles represent membrane-spanning β-strands of OmpA.

FIG. 2 shows the fractionation of membranes from JM109(pTX101) and JM109 cells. The percent of total membrane protein and β-lactamase activity in different fractions from a sucrose gradient of pTX101 is shown. β-lactamase activity was determined from the rate of hydrolysis of penicillin G. Fractions 2–7 had an average density of 1.22 g/cc, 11–13: p=1.19 g/cc and 16–20: p=1.15 g/cc corresponding exactly to the values for outer membrane, intermediate and inner membrane vesicles determined by Osborn.

FIGS. 3A and 3B show fractionation on a sucrose gradient of membranes from JM109(pTX101) and JM109(pJG311). FIG. 3A: Samples from every three fractions were pooled together and loaded in consecutive lanes. Lanes 2–8, pTX109: lanes 9–15, pJG311. Lanes: 1: Molecular weight markers: 2: fractions 1–3; 3: fractions 4–6; 4: fractions 7–9; 5: fractions 10–12; 6: fractions 13–15; 7: fractions 16–18; 8: fractions 19–21; 9: fractions 1–3; 10: fractions 4–6;11: fractions 7–9; 12: fractions 10–12; 13: fractions 13–15; 14: fractions 16–18; 15: fractions 19–21; 16: molecular weight markers. The molecular weight standards (BRL) are: myosin H-chain, 200 kDa: phosphorylase B, 97 kDa: bovine serum albumin, 68 kDa: ovalbumin, 43 kDa: and carbonic anhydrase, 29 kDa. Arrows indicate the fusion proteins Lpp-OmpA-β-lactamase (lane 2) and Lpp-β-lactamase (lane 9). FIG. 3B: Western blot of the JM109(pTX101) fractions from the sucrose gradient (FIG. 3A, lanes 2–8). The primary antibody was used at a concentration of 1:20.000. The gel was overloaded to show the presence of degradation products. There were no degradation products below the 32.000 dalton molecular weight standard. As with the native β-lactamase, the Lpp-OmpA-β-lactamase migrates as two bands depending on the oxidation of the single disulfide bond (30). The prestained molecular weight markers (Bio-Rad) have apparent molecular weights of: 106 kDa, phosphorylase B: 80 kDa, bovine serum albumin: 49 kDa, ovalbumin: 32 kDa, bovine carbonic anhydrase.

FIG. 4A shows a micrograph of JM109(pTX109) cells labeled with rabbit β-lactamase specific antibodies and rhodamine conjugated rabbit-specific antibodies viewed by fluorescence. FIG. 4B shows the same field of cells viewed by phase contrast microscopy.

FIG. 5 shows the effect of extended incubation on measured β-lactamase activity of the tripartite fusion expressed on the outer membrane surface of *E. coli*.

FIG. 6 shows a diagram of the plasmid pTX101.

FIG. 7 (SEQ ID NO:1) shows the DNA sequence of the genes coding for the tripartite fusion from pTX101.

FIG. 8 shows the condons for the segments included in the tripartite fusion from pTX101.

FIG. 9 shows a scanning electron micrograph of JM109(pTX101) cells labelled with anti-β-lactamase antibodies and secondary gold conjugated antibodies.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Materials and Methods

Bacterial Strains

E. coli strain JM109 (*endA*1 recA1 gyrA thi-1 hsdR17($r_k^-m_k^+$) relA1 supE44 X(lac-proAB) /F' traD36 proAB lacI^q lacZΔM15) (Invitrogen, San Diego, Calif.), ATCC 53323.

Escherichia coli strain JCB572 is obtained from Dr. J. Beckwith, Department of Molecular Biology and Molecular Genetics, Harvard Medical School, Boston, Mass. 02115 (Bardwell et al., 1991).

Plasmids

Plasmid pSWFII is prepared as described by Ehrmann et al., 1990.

Plasmid pJG311 (Ghrayeb and Inouye, 1984; Yamaguchi et al., 1988) was constructed by cutting pMH014 Cm^r (Yamaguchi et al., 1988) which contains the gene coding for the signal sequence and mature major outer membrane lipoprotein, with EcoRI and then removing all the lipoprotein gene except the signal sequence and the first nine amino acids. The β-lactamase gene, cut from pTG206, was ligated into this site creating pJG202. The region coding for the lipoprotein signal sequence, the first nine amino acids from the lipoprotein, and the entire mature β-lactamase, was transferred from pJG202 to the expression plasmid pIN-III-A-Cm^r (Yoshihiro et al., 1983) to create pJG311, which contains a Lpp-β-lactamase fusion. Plasmid pJG311 may also be obtained from Masayori Inouye, Department of Biochemistry, Robert Wood Johnson Medical at Rutgers University of Medicine and Dentistry of New Jersey, Piscataway, N.J. USA 08854.

Plasmid pRD87 is constructed in the same manner as pTU500 (Freudl et al., 1985). pTU500 is constructed by cutting the OmpA gene from pTU500/1 and ligating it into pUC9 (Vieira and Messing, 1982) thereby placing it under the control of the lac promoter. pRD87 was made identically except that the ompA gene from pTU500/1 was cloned into pUC8 (Vieira and Messing, 1982). The two plasmids, pTU500 and pRD87, are identical except that pTU500 contains an amber mutation at the seventh codon in the ompA sequence, while pRD87 does not contain the amber mutation. Plasmid pRD87 may also be obtained from Ulf Henning, Max-Planck-Institut fur Biologie, Correnssstrasse 38, D-7400 Tübingen, Germany.

Cultures

Cultures were grown in either LB medium (Difco) supplemented with 0.2% glucose or M9 medium supplemented with 0.2% casein amino acid hydrolysate and 0.2% glucose. The desired antibiotics were added as required.

General Procedures

SDS-PAGE was performed on 11% and 15% acrylamide gels. Protein samples, denatured for 5 minutes in boiling SDS containing β-mercaptoethanol, were loaded onto polyacrylamide gels and run at a constant current. The gels were stained with Coomassie brilliant blue (R 250) for 15 minutes and the background stain was removed overnight with a methanol/acetic acid destaining solution.

Western blots were performed by running 0.5 μg protein samples on polyacrylamide gels at constant current and were transferred overnight to nitrocellulose membranes. The membranes were incubated for 1 hour with rabbit anti-β-lactamase antibodies, rinsed, and incubated for 1 hour with horseradish peroxidase conjugated goat anti-rabbit antibodies. After further rinsing, the membranes were developed with 4-chloro-1-naphthol, which gives a distinct blue color at the sites containing horseradish peroxidase.

The enzymatic activity of β-lactamase was measured by the rate of hydrolysis of penicillin G or nitrocefin (Samuni, 1975; O'Callaghan et al., 1972). Hydrolysis of penicillin G gives linear decrease in the adsorption of light at 240 nm, while nitrocefin hydrolysis shows an adsorption increase at 482 nm. The changes in adsorption with time were measured in an LKB spectrophotometer. Protein concentrations were measured by the Bio-Rad assay using standard curves prepared from protein standards and comparing color developed with the reagent measured at 595 nm in a spectrophotometer.

The present invention relates to a novel chimeric gene from which a wide variety of recombinant expression vectors useful for surface expression of desired proteins is possible. Appropriately transformed gram-negative host cells will efficiently express proteins on the outer membrane surface without loss of inherent activity. A novel aspect of the fused gene is the use of

three separate DNA segments which act respectively (1) to target a fusion product to the host cell outer membrane and (2) to transverse the fusion product across the membrane to the outer surface where (3) the polypeptide expressed by a fused gene of interest becomes stably anchored to the surface.

The particular examples herein illustrated utilize recombinant vectors constructed from known DNA segments having particular functions. For example, various membrane proteins such as OmpA are known to contain both membrane targeting and transversing sequences. However, fusion of alkaline phosphatase with outer membrane proteins has not produced surface expressed alkaline phosphatase (Murphy et al., 1990). The present invention utilizes separate targeting and transversing domains. When engineered into a vector such that a gene for a desired polypeptide product is positioned downstream of both the targeting and transversing sequences, efficient surface expression of the product is effected. Moreover, the targeting sequence is positioned upstream of the transversing sequence.

It will be appreciated that the particular gene sequences shown here to construct a tripartite chimeric gene are not limited to deriving targeting and transversing sequences from lipoprotein and OmpA respectively. Other sequences with analogous function may be used. In particular, this invention may be efficiently practiced with the construct shown schematically in FIG. 6 and in particular detail in FIG. 7 (SEQ ID NO:1) illustrates useful targeting and transversing DNA sequences fused with the β-lactamase gene, although it is appreciated that numerous other polypeptide sequences could be used rather than β-lactamase.

The invention has numerous applications, a brief background for which is described.

Whole Cell Affinity Adsorbents

Affinity purifications of biomolecules rely primarily on the strong interactions between proteins and ligands. Typically, the ligand is bound to a solid support matrix which is employed in a chromatographic-type separation. More recently, suspensions of starch granules (Mattiasson and Ling 1986) or liposomes (Powell et al. 1989) have been used as supports for affinity purifications. In some of the most useful and specific separations, the affinity ligands are proteins such as antibodies, lectins or protein receptors (Mohr and Pommerening 1986, Turkova 1978). The preparation of protein affinity adsorbents involves the production, purification and the immobilization of the polypeptide on a solid support matrix. These three steps are generally complicated and often prohibitively expensive for large scale applications. On the other hand bacterial cells expressing proteins on their surface can serve as an important source of low cost solid phase adsorbents.

The human metallothionein gene protein has been expressed as a fusion with an outer membrane protein (Jacobs et al., 1989). Because of the way the fusion protein was constructed, metallothionein was localized on the internal side of the *E. coli* outer membrane, i.e., facing the periplasmic space. Nevertheless, since metal ions can diffuse through the outer membrane, the recombinant cells were able to bind as much as 66 fold more Cd^{+2} than normal *E. coli*. Another example of a high affinity cellular adsorbents (e.g., Kronvall et al. 1979) includes the use of cultured mammalian cells to remove viral impurities from blood samples (Tsao et al., 1988).

Whole Cells As Enzyme Carriers For Bioprocessing

The use of whole cells as enzymatic catalysts has been in use for several years. Typically, a microorganism which produces a certain enzyme is used as a biocatalyst, thus avoiding the costs associated with protein purification and immobilization steps. Usually the cells are first killed, treated with a permeabilizing agent to allow the diffusion of reactants and products into the cytoplasm and finally they are stabilized using some form of chemical crosslinking (Tampion and Tampion 1987). Several improvements on the preparation of whole cell biocatalyst have been made over the years. However, certain inherent limitations can not be overcome with the currently available technology. These are: i) The chemical methods which are used for permeabilization of the cell membrane can also result in deactivation of the important enzyme; ii) Other intracellular enzymes may compete for the reaction substrate giving rise to undesired byproducts and decreased yields; and iii) Intracellular degradation processes can limit the functional life of the biocatalyst. Clearly, all these problems can be eliminated if the enzyme is attached to the cell's exterior.

Live Bacterial Vaccines

Genetically weakened (attenuated) strains of bacteria that are able to survive and persist in the human or animal body can confer prolonged immunological protection against disease (Stover 1991). Non-recombinant live vaccines have been used for many years for large scale vaccinations (Dougan 1989). For example, live attenuated cultures of Baccillus Calmette-Guérin (BCG) which confer immunity against tuberculosis represent the most widely used vaccine in the world (Stover et al. 1991). Recently, emphasis has been shifted to the development of recombinant bacterial vaccines (Curtiss et al. 1989, Charles and Dougan 1990). In this case vaccination consists of the oral administration of a live culture of an attenuated enteric bacterium host such as *E. coli* or *Salmonella typhimurium* which expresses an antigenic peptide from a pathogen. Within the body, some of the bacteria find their way to the intestinal tract where they coexist with the wild type *E. coli* and other enteric microorganisms. In this way they ensure the presence of a low level of antigenic peptide in the body. Live vaccines provide more efficient immunity and longer protection against infections compared to subunit or killed bacterial vaccines. There are several reasons for the higher efficacy of live bacterial vaccines (Dougan et al. 1989): i) Protection correlates with how long the vaccine is present in the body (De Libero and Kaufman, 1986). Since the bacteria persist in the intestine for very long times, they are able to confer extended immunity; ii) Unlike most currently used vaccines, bacterial vaccines may be administered orally; and iii) Several antigens may be expressed simultaneously in bacteria thus giving rise to multipurpose vaccines.

Although the foregoing antigen may stimulate an immune response even when produced within the cell, the immunogenicity of peptide antigens can be greatly enhanced if they are expressed on the surface of an appropriate host strain (Taylor et al. 1990). This is because the surface of the bacteria such as Salmonella or *E. coli* acts as an adjuvant to enhance the immune response to the antigen. The most straightforward way to accomplish this is to insert the foreign peptide within a

surface exposed loop of an outer membrane protein which serves as the targeting signal. A fusion protein with the structure outer membrane protein--peptide--outer membrane protein is constructed and then the normal protein localization mechanism of the cell is exploited to carry the peptide to the surface. There appears, however to be an upper limit on the length of foreign polypeptides that can be inserted within outer membrane proteins. The maximum size of foreign sequence that can be accommodated within outer membrane proteins is around 45 to 50 amino acids (Newton et al. 1989, Charbit et al. 1988).

Several different outer membrane proteins have been exploited as targeting vehicles for the localization of foreign peptides (e.g., Charbit et al., 1988). A number of short amino acid sequences have been inserted within a surface exposed loop of the *E. coli* outer membrane protein maltoporin (LamB) (Charbit et al., 1988). The peptides were localized correctly so that the whole cells could be used to induce an immune response. Expression systems for the localization of antigenic peptides on bacterial surfaces have also been constructed using the *E. coli* K88ac and K88ad pilin proteins (Thiry et al. 1989), the *S. typhimurium* flagellin, (Newton et al. 1989) the TraT lipoprotein (Taylor et al. 1990) and the *E. coli* outer membrane porins PhoE, OmpA and OmpC (Agterberg 1987, Freundl 1989).

Prototype live bacterial vaccines have been prepared using cells having sequences from the influenza virus, cholera toxin B subunit and the gp 120 glycoprotein of HIV-1 expressed on their surface. However, the presence of a fragment of a protein from an infectious agent often does not give satisfactory protection against disease (Dougan et al. 1989).

An advantage of the present invention is the potential to express complete proteins from infectious agents on the surface of the carrier cells. Immunization with an intact protein is more likely to elicit a humoral immune response and provide protective immunity.

Vaccine Preparation and Use

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Pat. Nos. 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

Live bacterial vaccines are conventionally administered parenterally, by injection or in oral formulation. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10–95% of active ingredient, preferably 25–70%.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individual's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination. Suitable regimes for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be from two to twelve week intervals, more usually from three to five week intervals. The course of the immunization may be followed by assays for antibodies for the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Pat. Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

The invention also contemplates the use of disclosed nucleic acid segments in the construction of expression vectors or plasmids and use in host cells. The following is a general discussion relating to such use and the particular considerations in practicing this aspect of the invention.

Host Cell Cultures and Vectors

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example, strains such as *E. coli* K12 strain 294 (ATCC No. 31446), *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes are also preferred for expression. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325) or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various Pseudomonas species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells.

For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

The following examples are intended to illustrate the practice of the present invention and are not intended to be limiting. Although the invention is here demonstrated with βlactamase expressed on the surface of a cell membrane, numerous other proteins with various functions could be similarly expressed. These would include polypeptides with catalytic functions, metal binding capability and specific binding activity toward cell receptor sites. Moreover, the expression vectors and chimeric genes used therein may be constructed from a wide variety of targeting and transversing sequences, and are not limited to those derived from OmpA and Lpp.

EXAMPLE 1

The following example illustrates the construction of a recombinant vector encoding a desired protein, targeting and membrane translocating sequences. When used to transform suitable bacterial hosts, such a vector enables surface expression of active proteins, as shown here for the production of β-lactamase.

Construction of Plasmid pTX101 Containing Tripartite Chimeric Gene for β-Lactamase Expression

Plasmid pTX101 was prepared from pJG311, which contains the signal sequence and first 9 N-terminal amino acids of the mature major outer membrane lipoprotein of *E. coli* and the complete β-lactamase sequence. A unique EcoRI site in the linker region between the peptide region and the β-lactamase was cut with EcoRI. The cut plasmid was isolated from a low melting point agarose gel and the ends were made blunt using the Klenow fragment. pRD87, containing the OmpA gene, was simultaneously cut with HpaI and SphI, both unique sites, generating a 342 bp fragment containing the sequence for five of the eight outer membrane spanning domains of OmpA. This fragment was isolated from a low melting point agarose gel and made blunt with T4 DNA polymerase. The fragment, coding for amino acid residues 46–159 of OmpA, was ligated to the above pJG311 vector to make pTX101, which codes for the Lpp-OmpA-β-lactamase fusion. The ligation was transformed into *E. coli* strain JM109 made competent by the rubidium chloride method.

The Lpp-OmpA-β-lactamase was expressed from the strong lpp promoter which is inducible by IPTG (isopropyl thiogalactoside). Plasmid pTX101 also carries the lacI repressor. Although induction with IPTG resulted in high levels of protein production which are lethal to the cell, good expression was nevertheless obtained in the absence of inducer. Cultures were harvested in late exponential phase and the cells lysed and separated into soluble and cell envelope fractions by high speed centrifugation. Approximately 84% of total β-lactamase activity from JM109(pTX101) lysates was found in the cell envelope fraction. Essentially all the remaining activity was present in the soluble fraction of the cell lysates with less than 0.5% in the extracellular fluid. Even after prolonged incubation of stationary phase cells (24 hrs) there was no increase in the percentage of β-lactamase in the extracellular fluid, indicating that the fusion protein was not released from the cells, Figure 5. Qualitatively similar results were observed when the distribution of the fusion protein in the different fractions was examined by immunoblotting with β-lactamase or OmpA-specific antisera. The Lpp-β-lactamase protein from plasmid pJG311 had 2-fold higher total activities compared to the three-part fusion which contained the OmpA insert. This protein was also found predominantly in the membrane pellet (98% of total activity).

EXAMPLE 2

This example illustrates that a heterologous polypeptide prepared as in Example 1 is exposed to the external medium and retains activity.

Cell Fractionation

First it was demonstrated that the fusion protein was localized to the outer membrane. Cells were harvested from 200 ml of LB medium containing 0.2% glucose at an $A_{600}=1.0$, washed in 25 mM Tris-HCl (pH 7.4) and resuspended in 10 ml of the same buffer containing 1 mM EDTA and 100 μg/ml lysozyme at 4° C. After a 2 minute incubation the cells were lysed by two passages through a French pressure cell at 10,000 psi. The cellular debris was removed by centrifugation at 2,500×g for 8 minutes and the total membranes were spun down by centrifugation at 115,000×g for 1 hour. Membranes were resuspended in 0.8 ml of Tris-HCl buffer containing 25% sucrose and loaded onto a step gradient of 20, 35, 40, 45, 50 and 55% (w/w) sucrose. After centrifugation at 165,000×g for 16 hours in a Beckman SW41Ti rotor, 0.5 ml fractions were collected from the bottom of the tube. The density of the fractions was determined from refractive index measurements. The concentration of sucrose was lowered to <10% (w/w) by diluting the samples with Tris-HCl buffer followed by centrifugation to pellet the membranes.

Two distinct protein peaks were obtained in fractions having the expected densities for inner and outer membrane vesicles (Osborn et al., 1972). Virtually all the β-lactamase enzymatic activity was found in the higher density fractions which corresponded to the outer membrane vesicles, FIG. 2. A protein band migrating at a molecular weight of approximately 43,000 daltons, the expected size of Lpp-OmpA-β-lactamase band was found and it was comparable in abundance to that of the major outer membrane proteins, FIG. 3A. The fusion protein was subjected to some degradation resulting in the appearance of lower molecular weight bands that crossreacted with β-lactamase-specific antibodies in immunoblots, FIG. 3B. The relatively small proportion

of degradation fragments indicated that most of the fusion protein was not subjected to proteolysis.

Exposure of β-Lactamase on Cell Surface

Localization of the β-lactamase domain with respect to the external surface of *E. coli* was determined by various immunocytochemistry methods, activity assays and protease accessibility experiments. For immunofluorescence determinations, whole cells were labelled with rabbit β-lactamase-specific antibodies followed by secondary rhodamine-conjugated goat anti-rabbit antibodies. Washed, mid-exponential phase cells were resuspended in phosphate buffered saline (PBS) with or without 0.1 mg/ml trypsin and incubated at 37° C. Soybean trypsin inhibitor was added at different times to stop the reaction and incubation at 37° C. was continued for a total of 1 hour. All subsequent procedures were conducted at room temperature. The cells were washed with PBS, incubated for 45 minutes with PBS and 1% bovine serum albumin, washed and then incubated in the same PBS/BSA solution with rabbit anti-βlactamase antibodies at 1:1,000 dilution for 45 minutes. Following another three washes with PBS/BSA, the cells were mixed with rhodamine-conjugated goat anti-rabbit antibodies, incubated for 45 minutes and then washed three more times. Finally, the cells were resuspended in PBS and examined by phase contrast and video enhanced fluorescence microscope.

In control experiments, no fluorescence above background was detectable with JM109(pJG311) cells expressing the Lpp-β-lactamase fusion protein, indicating that there was no exportation to the outer surface. FIGS. 4A and 4B show a comparison of the same field of JM109(pTX101) cells viewed with fluorescence and phase contrast microscopies. Nearly all the cells became fluorescent, indicating sequences recognized by the anti-β-lactamase antibodies. Incubation with trypsin for various times prior to antibody labelling resulted in a gradual decrease in the fluorescent signal. After 1 hour of incubation no signal was detected.

For immunoelectron microscopy cells were labelled with rabbit anti-β-lactamase specific antibodies, washed in various buffers as described above for the immunofluorescence experiments and reacted with secondary 30 nm diameter colloidal gold conjugated goat anti-rabbit antibodies. The labelled cells were positively stained with uranyl-acetate and viewed by scanning electron microscopy. In control experiments, no labelling occurred with JM109(pJG311) cells. FIG. 9 shows JM109(pTX101) cells so labelled, indicating the presence of sequences recognized by the anti-β-lactamase antibodies on the external surface.

The presence of enzymatically active β-lactamase on the cell surface was determined from protease accessibility experiments and the rates of hydrolysis of substrates not readily diffusible through the outer membrane. Cultures grown in M9 medium were harvested at A_{600}=1.0, washed with fresh medium and resuspended in M9 salts without glucose or antibiotics. The β-lactamase activity in the whole cells was determined using nitrocefin and penicillin G as substrates. The cells were incubated for 1 hr at 37° C. in the presence or absence of 0.1 mg/ml of either proteinase K or trypsin. The protease digestions were stopped by adding 10 mM phenylmethylsulfonyl fluoride or 0.2 mg/ml soybean trypsin inhibitor respectively. Subsequently, the cells were lysed and centrifuged at 2500×g for 8 min to remove unbroken cells. The membranes were pelleted as described above, resuspended in 50 mM potassium phosphate buffer, pH 6.5 and the remaining enzymatic activity measured.

In cells containing plasmid pTX101, approximately 20% of the total membrane-bound activity was reproducibly lost after a one hour incubation with either trypsin or proteinase K, compared with only a 3% decrease in JM109(pJG311), Table 1. A comparable, somewhat higher percentage of surface exposed activity was obtained from the rates of hydrolysis of nitrocefin in intact and lysed cells. Nitrocefin does not cross the outer membrane of *E. coli* and therefore can be used to test activity of extracellular β-lactamase (Kornacker and Pugsley, 1990). The rate of hydrolysis of nitrocefin by intact cultures of JM109(pJG311) was in agreement with results of protease accessibility studies, indicating Lpp-β-lactamase is not transported across the cell surface. Approximately 20–30% of the enzymatic activity of Lpp-OmpA-β-lactamase was surface exposed on cells grown at 37° C., a significant increase over the background in control cultures, see Table 1.

TABLE 1

Percent Surface Exposed β-Lactamase as Determined by Protease Accessibility and Enzymatic Activity Using Nitrocefin

Plasmid	Temperature	Percent decrease in penicillin G hydrolysis following incubation with: Proteinase K[a]	Trypsin[a,b]	Nitocefin activity in intact cells as percentage of total activity in membranes[b]
pJG311	37° C.	3	3	6
pTX101	37° C.	23	18	38
pTX101	24° C.		89	81

[a]Cells were incubated with proteinase K or trypsin for one hour and the total membrane fractions were isolated as described in the materials and methods sections. The percent of exposed β-lactamase corresponds to the activity remaining after incubation with proteases relative to untreated cells.
[b]The standard deviation for all experiments was less than ±5% of the reported mean values.

EXAMPLE 3

The following example demonstrates that even after extended incubation, the region of the tripartite fusion containing the target protein remains stably anchored to the outer membrane of the host cell. This example demonstrates the surface stability of surface-expressed fusion polypeptides using the disclosed methods. Surface Stability of Translocated Fusion Protein β-Lactamase

JM109 cells with the plasmid pTX101 were grown in LB supplemented with glucose and ampicillin. After 4, 6, 8 and 24 hours, 10 ml samples were collected and separated into culture supernatant, soluble and membrane fractions, FIG. 5. The cells were first pelleted by centrifugation at 8,000×g and the resulting supernatant was saved as the culture supernatant fraction. The pelleted cells were resuspended in 50 mM potassium phosphate buffer (pH 7) and lysed in a French pressure cell at 20,000 psi. The lysed samples were centrifuged at 2,500×g to pellet any unlysed cells and then centri-

fuged for 1 hr at 115,000×g. The supernatant from the high speed centrifugation was removed and saved as the soluble fraction and the pelleted membranes resuspended in 50 mM potassium phosphate buffer to obtain the membrane fractions. β-lactamase activity performed on the fractions indicated that even after prolonged incubation (24 hrs) the fusion was stably anchored to the outer membrane (FIG. 5).

EXAMPLE 4

This example illustrates that efficient surface-expression and maintenance of activity of surface-expressed polypeptides is affected by the culture temperature. The example is illustrated with β-lactamase, but effective surface expression with maintenance of function is also affected by temperature for alkaline phosphatase.

Effect of Temperature on β-Lactamase Expression and Activity

Cultures grown at 24° C. exhibited almost quantitative β-lactamase activity on the cell surface. The rate of nitrocefin hydrolysis and trypsin accessibility indicated 80–87% surface exposure, Table 1.

EXAMPLE 5

The following example illustrates expression of alkaline phosphatase on the outer membrane surface of *E. coli*. Alkaline phosphatase is a large dimeric enzyme with a monomer size of approximately 43,000 D. Disulfide bonds form rapidly after the protein has been exported from the cytoplasm. The expression of active protein on the bacterial surface indicates that there is no significant effect on the protein's ability to retain or fold to its native form after membrane translocation. This example also illustrates the versatility of the method in that alkaline phosphatase is a relatively large protein. In this example, tertiary and quaternary structures are unaffected by the expression.

Expression of Alkaline Phosphatase on *E. coli* Cell Surface

Plasmid pTX101 was cut with EcoRI at the unique site in the linker region between the OmpA and β-lactamase. Subsequently the DNA was treated with the Klenow fragment of DNA polymerase to create blunt ends. The phoA gene coding for the sequence of alkaline phosphatase was isolated from the plasmid pSWFII. A DNA fragment containing the phoA gene was obtained by cutting pSWFII with SbaI and then blunt-ended using the Klenow fragment. The linearized pTX101 vector and the phoA gene fragment were ligated overnight and the DNA was transformed into *E. coli* strain JM109. The new plasmid encoding the Lpp-OmpA-PhoA tripartite fusion was designated pTX1000.Exposure of the alkaline phosphatase on the surface of *E. coli* was tested by immunofluorescence microscopy using anti-alkaline phosphatase antibodies.

The degree of localization of alkaline phosphatase on the cell surface is expected to be enhanced in the strain JCB572 (Bardwell et al., 1991) which is deficient in the gene for the *E. coli* periplasmic protein disulfide isomerase, DsbA, and in cultures incubated at sub-optimal growth temperatures, for example 24° C.

PROPHETIC EXAMPLE 6

The present example outlines the procedure contemplated as useful for expressing an antibody on the surface of *E. coli*. The antibody used for illustration is a catalysic antibody capable of catalysis in addition to binding its cognate antigen. Although this example is illustrated with antibody 37C4 against a particular hapten, other high affinity antibodies could be surface-expressed in a like manner. The example shows how the disclosed methods could be used to prepare a single-chain F_v, that is, a recombinant protein composed of a V_L chain linked to a V_H chain with a polypeptide linker. This particular ScF_v is a catalytic antibody.

Expression of Single-Chain F_v Antibody on *E. coli* Cell Surface

Antibody 37C4 exhibits high binding against the hapten tris(4-methoxyphenyl)phosphonium (dissociation constant $>10^{-10}M^{-1}$. The antibody acts as a catalyst for the cleavage of various trityl ethers, increasing the reaction rate by about 200 fold compared to the uncatalyzed reaction in the absence of antibody. Total mRNA from the 37C4 hybridoma line is isolated and purified by standard techniques (Ausubel et al. 1987). The purified mRNA is used as a template for cDNA synthesis using a polymerase chain amplification technique (Sastry et al. 1989). The V_L and the V_H domains of the 37C4 antibody are cloned using suitable primers designed to introduce an in-frame EcoRI restriction site at the N-terminus of the V_H and another one at the carboxy terminus of the V_L for easy subcloning of the F_v gene into the surface expression vector pTX101. Plasmid pTX101 contains a unique EcoRI site located at the downstream end of the DNA sequence for the OmpA domain and immediately before the beginning of the β-lactamase gene. An Lpp-OmpA-scF_v tripartite fusion is constructed by digestion of pTX101 with EcoRI and ligation of the scF_v fragment. The resulting plasmid is transformed into *E. coli* strain JM109. The presence of the single-chain antibody on the cell surface allows the cells to bind to a complex of tris(4-methoxyphenyl)phosphonium antigen linked to the protein avidin. The antigen is linked to avidin via its carboxy terminus by standard techniques (Staros et al. 1986). Finally, avidin is detected by immunofluorescence microscopy using anti-avidin antibodies conjugated to fluorescein (obtained from Vector Laboratories, Burlingame, Calif. Cells expressing the scF_v fragment give a fluorescence signal whereas control *E. coli* do not.

PROPHETIC EXAMPLE 7

The present example outlines the procedure contemplated as useful for the selection of antibodies with high antigen binding affinity. The method is based on a selection procedure for recombinant antibody fragments on the surface of *E. coli*.

The method illustrated will overcome many of the problems currently associated with display of antibody molecules on phage surfaces. In particular, subcloning will not be necessary for production, the number of antibodies on the cell surface can be controlled and the greater flexibility in the design of the expression system will help ensure proper folding.

Selection of High Antigen Binding Affinity Antibodies Using a Cell Surface Display System

BALB/C female mice (6–8 weeks old) are immunized with the hapten tris(4-methoxyphenyl)phosphonium coupled to Supercarrier (Pierce Chemical, Chicago, Ill.) dissolved in Freund's complete adjuvant administered intraperitoneally at a dose of 1 mg per animal. Follow up injections are given intramuscularly once per

week for three weeks, rested 2 weeks and given a booster shot before checking for antibody production. Incomplete Freund's adjuvant is used for all injections subsequent to the first injection.

A library of single-chain F_v antibodies is constructed using a polymerase chain reaction with total spleen mRNA from the immunized mice (Clarckson et al., 1991). The PCR primers are designed to introduce an in-frame EcoRI restriction site at the N-terminus of the V_H and another restriction site at the carboxy terminus of the V_L for easy subcloning of the F_v gene into the surface expression vector pTX101. Subsequently, the library DNA is digested with EcoRI, a gene fragment of approximately 730 base pairs containing the entire scF_v gene is identified. This DNA fragment is isolated and ligated to EcoRI digested plasmid pTX101. The ligation mixture is transformed into competent *E. coli* cells and transformants are selected on LB plates containing the antibiotic chloramphenicol. Plasmids in which the scF_v is inserted in the correct orientation result in expression of tripartite fusion proteins in the order (from the amino terminus): Lpp-OmpA-scF_v. Colonies are pooled from the plate and grown in rich media at 23° C. to allow efficient localization of the scF_v to the cell surface. Approximately 10^9 cells are diluted in buffer to halt further growth and are loaded onto an affinity column (approx 5 ml bed vol) containing the immobilized hapten tris(4-methoxyphenyl)phosphonium. The column is washed with Tris-HCl buffer, pH 7.0. Bound cells are eluted by applying a linear gradient of the hapten. Elution of the cells from the column is directly related to the binding affinity of the exposed antibodies. This results in enrichment of high-binding affinity antibodies expressed on the surface of cells. The cells are collected, grown and used to prepare antibodies.

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The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for or teach methodology, techniques and/or compositions employed herein.

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SEQUENCE LISTING

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(i i i) NUMBER OF SEQUENCES: 2

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(A) LENGTH: 1273 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(A) LENGTH: 1273 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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35                  40                  45                  50

AGC GTT GAA AAC GGT GCA TAC AAA GCT CAG GGC GTT CAA CTG ACC GCT AAA  204
Ser Val Glu Asn Gly Ala Tyr Lys Ala Gln Gly Val Gln Leu Thr Ala Lys
            55                  60                  65

CTG GGT TAC CCA ATC ACT GAC GAC CTG GAC ATC TAC ACT CGT CTG GGT GGC  255
Leu Gly Tyr Pro Ile Thr Asp Asp Leu Asp Ile Tyr Thr Arg Leu Gly Gly
      70                  75                  80                  85

ATG GTA TGG CGT GCA GAC ACT AAA TCC AAC GTT TAT GGT AAA AAC CAC GAC  306
Met Val Trp Arg Ala Asp Thr Lys Ser Asn Val Tyr Gly Lys Asn His Asp
                  90                  95                 100

ACC GGC GTT TCT CCG GTC TTC GCT GGC GGT GTT GAG TAC GCG ATC ACT CCT  357
Thr Gly Val Ser Pro Val Phe Ala Gly Gly Val Glu Tyr Ala Ile Thr Pro
            105                 110                 115

GAA ATC GCT ACC CGT CTG GAA TAC CAG TGG ACC AAC AAC ATC GGT GAC GCA  408
Glu Ile Ala Thr Arg Leu Glu Tyr Gln Trp Thr Asn Asn Ile Gly Asp Ala
120                 125                 130                 135

CAC ACC ATC GGC ACT CGT CCG GAC AAC GGA ATT CCG GGT CAC CCA GAA ACG  459
His Thr Ile Gly Thr Arg Pro Asp Asn Gly Ile Pro Gly His Pro Glu Thr
                  140                 145                 150

CTG GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA GTG GGT TAC  510
Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr
      155                 160                 165                 170

ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC GAA  561
Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu
                  175                 180                 185

GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGC GCG GTA  612
Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val
            190                 195                 200

TTA TCC CGT GTT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC ATA CAC TAT  663
Leu Ser Arg Val Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr
205                 210                 215                 220

TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA AAG CAT CTT ACG  714
Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
                  225                 230                 235

GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC ATA ACC ATG AGT GAT  765
Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser Asp
      240                 245                 250                 255

AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC GGA GGA CCG AAG GAG CTA  816
Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys Glu Leu
                  260                 265                 270

ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT GTA ACT CGC CTT GAT CGT TGG  867
Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu Asp Arg Trp
            275                 280                 285

GAA CCG GAG CTG AAT GAA GCC ATA CCA AAC GAC GAG CGT GAC ACC ACG ATG  918
Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg Asp Thr Thr Met
290                 295                 300                 305

CCT GCA GCA ATG GCA ACA ACG TTG CGC AAA CTA TTA ACT GGC GAA CTA CTT  969
Pro Ala Ala Met Ala Thr Thr Leu Arg Lys Leu Leu Thr Gly Glu Leu Leu
                  310                 315                 320

ACT CTA GCT TCC CGG CAA CAA TTA ATA GAC TGG ATG GAG GCG GAT AAA GTT 1020
Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp Met Glu Ala Asp Lys Val
      325                 330                 335                 340

GCA GGA CCA CTT CTG CGC TCG GCC CTT CCG GCT GGC TGG TTT ATT CGT GAT 1071
Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro Ala Gly Trp Phe Ile Ala Asp
                  345                 350                 355

AAA TCT GGA GCC GGT GAG CGT GGG TCT CGC GGT ATC ATT GCA GCA CTG GGG 1122
Lys Ser Gly Ala Gly Glu Arg Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly
            360                 365                 370

CCA GAT GGT AAG CCC TCC CGT ATC GTA GTT ATC TAC ACG ACG GGG AGT CAG 1173
Pro Asp Gly Lys Pro Ser Arg Ile Val Val Ile Tyr Thr Thr Gly Ser Gln
375                 380                 385                 390
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GCA ACT ATG GAT GAA CGA AAT AGA CAG ATC GCT GAG ATA GGT GCC TCA CTG 1224
Ala Thr Met Asp Glu Arg Asn Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu
                395                 400                 405

ATT AAG CAT TGG TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGA          1273
Ile Lys His Trp
     410      412
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What is claimed is:

1. A recombinant DNA for expressing a polypeptide stably anchored on the external surface of the outer membrane of *E. coli* or Salmonella comprising:

(A) A Salmonella or *E. coli* lipoprotein 5' gene segment which encodes at least the signal peptide and at least the first three amino acids of the mature protein;

(B) A DNA segment encoding a transmembrane outer membrane protein selected from the group consisting of OmpA, OmpC, OmpF and OmpT of *E. coli* or Salmonella; and

(C) A DNA segment encoding a desired soluble heterologous or homologous polypeptide not normally found in the outer membrane of gram negative bacteria wherein DNA (A) is linked 5' to DNA (B) and DNA (B) is linked 5' to DNA (C), all operatively linked with a promoter sequence to express and anchor the desired polypeptide on the external bacterial surface.

2. A recombinant DNA vector comprising:

An *E. coli* or Salmonella lipoprotein 5' gene segment which encodes the signal peptide and at least the first three amino acids of the mature protein;

A DNA segment encoding a transmembrane outer membrane protein selected from the group consisting of OmpA, OmpT, OmpF and OmpC of *E. coli* or Salmonella; and

A polylinker DNA segment into which a DNA encoding soluble heterologous or homologous polypeptide not normally found in the outer membrane of gram-negative bacteria may be inserted, all being operatively linked 5' to 3' with a promoter sequence to express and stably anchor the polypeptide to the external bacterial surface.

3. The recombinant DNA of claim 1 or the recombinant vector of claim 2 wherein the lipoprotein gene segment comprises the 5' segment of a gene selected from a group consisting of osmB, traT, NlpB, and Psuedomonas lipoprotein 1.

4. The recombinant DNA of claim 1 or the recombinant vector of claim 2 wherein the DNA encoding the lipoprotein gene segment encodes the N-terminal amino acid residues of FIG. 7 (SEQ ID NO. 1) at base pair positions 1–87.

5. The recombinant DNA of claim 1 or the recombinant vector of claim 2 wherein the transmembrane protein domain comprises an amino acid sequence of FIG. 7 (SEQ ID NO. 1) at base pairs 94–435.

6. The recombinant DNA of claim 1 or the recombinant vector of claim 2 wherein the promoter is an inducible promoter.

7. The recombinant DNA or the recombinant vector of claim 6 wherein the inducible promoter is Lpp or lac promoter.

8. An *Escherichia coli* transformed with a vector containing the DNA of claim 1.

9. A Salmonella transformed with a vector containing the DNA of claim 1.

10. A method of preparing a functional polypeptide stably anchored on the external surface of the outer membrane of a bacterial cell comprising growing the bacterial host cell of claim 8 or claim 9 under conditions permitting DNA expression and protein production followed by recovering the stably anchored polypeptide so produced.

11. The method of claim 10 wherein the growing is conducted between about 22° C. and 40° C.

12. The method of claim 10 wherein the growing is conducted at about 24° C.

13. The recombinant DNA of claim 1 wherein the soluble homologous polypeptide is β-lactamase or alkaline phosphatase.

14. The recombinant DNA of claim 1 wherein the heterologous polypeptide is a single chain antibody or antibody fragment.

15. A kit for use in preparing transformed *E. coli* or Salmonella comprising an expression vector that includes the recombinant DNA of claim 1.

16. The kit of claim 15 wherein the expression vector has the sequence shown in FIG. 7 (SEQ ID NO: 1).

17. The kit of claim 15 wherein the expression vector is provided in lyophilized form or in a suitable buffer.

18. The recombinant DNA of claim 1 wherein the heterologous polypeptide is cellulose binding domain of cellulase.

19. The recombinant DNA of claim 1 wherein the encoded transmembrane protein is a transmembrane sequence of OmpA.

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